SOCIETY OF TOXICOLOGY

MARCH 10-14, 2013

San Antonio, TX

Course: Toxic Effects of Metals

Presentation: Introduction

Speaker: Dr. Michael Waalkes

DR. WAALKES: Okay. If we could get going here. I'd like to welcome you to SOT's annual meeting. I am actually the acting Continuing Education Committee Liaison because the liaison could not make it. His trip was cancelled because of the rescission. I'm also the council contact to Continuing Education. My name is Michael Waalkes. I'm also a speaker. So there you go. A lot of hats here.

We have a very exciting course planned for you today. I hope you'll appreciate it. It is AM07 Toxic Effects of Metals. And if you're not supposed to be going to this city, you should be on a different plane.

So let me remind you, please, to turn off your phones or put them on mute or vibrate, or whatever you do to keep them quiet. And please fill out the survey that will be e-mailed to you in the next couple of days. We really put a lot of credence into that and help formulate our next Continuing Education courses.

And on a good side here, good note here, we'll have a coffee break at 10 to 10:30 right outside here. So that's the housekeeping liaison stuff.

Now, let me briefly introduce the topic here for you. And, again, I'm Michael Waalkes, and I'm introducing this topic.

Metals are widely used or widely distributed in the natural environment. They're not biodegradable and they're very persistent. And there's been global dispersion because of human use. Humans have used a lot of different metals for thousands of years, and in fact it was recorded back as far as 4000 BC that nine metals were in active use by man. But most metals have been discovered and used since the onset of the industrial revolution. And so, human exposure to metals is essentially inevitable. But metal use is a

key to human civilization and the progress of human civilization. They're intensely utilized in modern society. You take metals away from the chair that you're sitting in, you would be sitting on the floor essentially. They serve many indispensable functions. Most are rarely recycled. There's clearly notable exceptions, and we're getting better at this as we go along. The form of metals change, but the basic unit is neither created nor destroyed by human endeavors, and this is important to keep in mind. They just move around and typically they concentrate in the biosphere.

And here's an example of human activities in toxic metals in the environment. This is lead. Lead was used in increasing amounts at the onset of the industrial revolution and then we added it to gasoline and it spiked up. We've taken it out and it started going down. This is lead in Greenland Ice. So you can see that these metals are very persistent in the biosphere.

Now, they pose serious risks to human health, and that's what the rest of the day or rest of the morning will be about. Many metals are on tops of the

CDC's list of priority hazards to humans. Arsenic is consistently number one. Lead is up there. Mercury, cadmium, hexavalent chromium. So we have a real murderer's row here. Metals as toxicants have caused major poisonings in human history, including Minamata's disease, which was a very nasty neurological disease from eating fish contaminated with methylmercury from industrial wastewater, ouch-ouch disease, which is how this is translated to English, which was severe osteoporosis by cadmium from contaminated rice water, and lead in the Roman Empire, which probably led to its downfall, which was one of the first food additives they added to cheap wine to make it taste sweeter. Natural arsenic in well water, which is a current major issue in Asia where we have 1.5 million people being poisoned and getting cancer.

And that's it for me. And I don't think there will be any questions, so I would like to introduce Mike Hughes who will talk about general metals toxicology. Thank you very much.

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Presentation: Essentials of Metals Toxicology

Speaker: Dr. Michael Hughes

P R O C E E D I N G S

DR. HUGHES: Thank you, Mike. Again, my name is Mike Hughes. If you have any questions and you don't have time to ask them today, my e-mail address is right there. So just feel free to contact me.

Here's our outline. We're going to talk a little bit about the physical and chemical properties, how metals are essential. ADME is the absorption, distribution, metabolism and elimination. Say a few words about toxicity mechanisms and things that can influence its toxicity, and a few words about chelation.

Metals have physical and chemical characteristics. The general elemental forms just have a metallic luster; they can conduct electricity, and heat, and they're malleable and ductile. “Malleable” means that you can take a hammer to them and just pound them and they'll flatten out, they won't break apart. “Ductile” means that you can form them into wires such as copper wires. But for toxicology, we're most interested in the chemical properties.

The general definition of a metal is that under biological conditions it is an element that can lose one or more electrons. Metals may also form basic oxides. If there's one thing you get out of this talk today, you always want to remember the term chemical species. People tend to talk about in general about metals and they'll just throw out the words, lead and arsenic. If you're reviewing papers or talking to your colleagues about metals, ask them what the chemical species is of the metal, because they may differ in toxicity. The definition of chemical species is the specific form of an element. You want to include the isotopic composition, electronic or

oxidation state, and its complex molecular structure. Examples are chromate and cadmium chloride.

Here are some terms called metalloid or semimetal, and this is an element that has a mixture of both metal and non-metal properties. Now, depending on who you read, there are different metalloids. The most familiar ones are arsenic, selenium and germanium. You might hear terms called heavy metals. I have no idea what a heavy metal is. Here I show a chart of the periodic table, and you can see where the heavy metals are located. It's better to talk about your metal in specific terms. Heavy metals generally describe transition metals, but there are over 50 transition metals. There's an interesting article that I included in the references by Duffus.,He is critical of the use of the term "heavy metals." He just doesn't like it, but he's a chemist, so --

Just a little background information here on this slide. Remember, we have orbitals where you find electrons. There are different orbitals, s, p, d, f, g. The s orbitals, – there are different energy levels, and there's one to two electrons per orbital.

Here we have s1 s2, p has three orbitals, x, y, z; d has 5. I only have 3 shown here; f has 7. So just recall from your basic chemistry, the electronic configuration for hydrogen is 1s1. The second "1" being the electron. Chromium goes out to 3d6. And then for things that are more complex, instead of extending out this whole configuration, you can go down to the noble elements, write for example, Xenon, and then just fill out the rest of the electronic configuration.

Here's the periodic table. I think the most striking thing is how many metals there are in the environment. I have placed an X on thenon-metals. I have circled in blue what people say are the metalloids. In some readings you're going to see boronis a non-metal. It depends on who you read. Silicon, arsenic is definitely one. I have here a question mark for selenium, but if you talk to most of the people that work with selenium, they're going to tell you it's a metalloid. But some of these still are common or commonly accepted as metalloids or sometimes they're -- like the ones in the dash lines are --

there's a big question. You can break up the periodic table into different blocks. This would be the S block and includes the alkaline metals and alkaline earth. These would have one electron in the S orbital. S orbital is completely filled (in the alkaline earths). You have the P block Which includes metals like gallium and lead. Then you have the D block. These are the transition metals. Remember I was talking about people calling these heavy metals, but you can see there are a whole lot of metals here, so it's better to talk about specific species. Then you have the D block. This is where these are partially-filled electrons in the D block for this group. And then the F block here, the lanthanides and actinides, they have partially-filled F orbitals. The actinides are naturally radioactive.

You're going to hear some talks about oxidation number or state. That's the charge of an atom within an ion or molecule would have if the whole ion or molecule was composed entirely of ions.

So a couple rules. Basically the free element itself has an oxidation state of zero. For example, mercury vapor. The hydrogen is suggested to have one

or plus one. Oxygen is negative two. If you have a neutral compound, the whole thing is zero, that is, the whole molecule itself. So again, element manganese is zero. Here we have permanganate. The whole molecule is minus one. That's the overall charge. From our rules here, oxygen is minus two. We have four minus twos; so four times minus two is minus eight, and we know the whole charge is minus one, so manganese is seven.

There's some nomenclature you'll hear with these endings on the name of the metals or the -- the molecule. You have "ous" or "ic". “ous” would be the lower oxygenation state. "ic" would be higher, so for example, ferrous/ferric, mercurous/mercuric; and then "ite" and "ate". These are arsenite and arsenate. So arsenite is a lower oxidation state.

Some properties, metal ions lose electrons from their outer shells. It's easier to form a metal ion in a liquid than a gas and that's because of the energy of hydration. Cations can form a sphere of water around them with the dipole from the water to take out this -- to lessen the charge here. It's kind

of interesting; you can get spheres of water molecules all around a cationic metal. It may go out to several spheres.

We said the metals lose electrons, but not equally -- it's not across the board the same gain or loss of energy. For some metals, it's easier to lose electrons or gain than in others.

For metals basically to be toxic, they need to be soluble. Solubility is a major factor influencing the availability and absorption of metals. And it depends on your media. For example, water versus biological fluid, pH can affect it, the metal oxidation state, presence of other ions. Generally the soluble inorganic compounds include the nitrates and the chlorides and others . Some are insoluable such as the phosphates and carbonates.

Metals can form ionic and covalent bonds, and you have a whole host of different types of compounds. For example, you have lithium hydride, silver chloride, calcium chloride. Metals can form complexes such as cisplatin and calcium EDTA. EDTA is a chelator, as you can see it coordinates the metal here.

You have the organometallic compounds like dimethylmercury. MMT is a gas additive. Arsenobetaine is an interesting arsenic compound that you find in shrimp. I guess I'll digress here, talk about chemical species.

A colleague of mine, we were working with arsenic in our lab. She had a physical at work and the physician decided they were going to check her urine because we were working with arsenic. It came back that she has been exposed to arsenic. Checking urine is a biomarker of exposure for arsenic. They looked at the total arsenic. So of course the safety people were worried and they started asking her a couple of questions. Well, it turned out two days before she had her physical, she had shrimp. What happens is that shrimp is loaded with arsenobetaine, and you absorb it, and then you pee it right out. They did total arsenic levels. That's why it's really important that you talk about chemical species. If they would have speciated her urine, then they would have found, oh, wow, we have a lot of arsenobetaine. It's relatively non-toxic compared to the inorganic arsenic.

Some metal-containing biomolecules, we have the metalloporphyrins such as hemoglobin and cytochromes. Proteins can carry metals: Transferrin, ferritin. Metalloenzymes: Superoxide dismutase. This is a very important enzyme. It degrades superoxide. And there are different metals found with different superoxide dismutases.

Metals are essential for human life. This is iron, zinc, copper, manganese, molybdenum, and cerium. You might read that chromium is too. There are some questions now if chromium is or is not an essential metal. The metals are important in a biochemical process. Basically, if you take it out of the diet, some functionalor structural abnormality will occur. If you put it back in the diet, it will revert back to the normal status.

So here's a chart . The X axis is the total dairy intake. You have always some normal homeostatic level. If you decrease or are deficient, you start to have a risk of toxicity. Examples of deficiency for metals are anemia for iron; selenium Keshan disease; and zinc, poor immunity and growth failure. But if you

get excessive metals, again, you have risks; you can get GI distress and cirrhosis for iron, and GI distress hematuria and jaundice for copper.

Let me say a few words about the disposition of metals. Metals can be in a vapor phase. And I believe -- you might have a -- on the -- below the temperature line, you might have the phrase “particles in a gas”. Just please strike that out. That's a typo on my part.

A vapor is a substance in a gas phase at a temperature below its critical phase. Examples are mercury vapor and nickel carbonate. These are absorbed through passive diffusion. Some determinants are water solubility, tissue reactivity, blood-to-gas partition coefficients. There is this illness called metal fume fever. It happened with welders if they're not in a well-ventilated area. The metal vapors can accumulate and react with oxygen to form a metal oxide, and they can get acute flu-like symptoms.

Metals are also found in aerosols.An aerosol is a suspension of solid or liquid particles in a gas. Examples, tobacco smoke, you can find these particles,

lead, nickel, arsenic, coal fly ash also has metals. Particle size is a really important determinant of where the particles go into the lungs. The smaller the size of the particle, deeper into the lung they'll penetrate. Water solubility is really important, because if it's highly water soluble the particles are going to be up in the nasal terminates or perhaps the bronchus. The mechanisms of particle deposition are impaction, sedimentation and diffusion.

The particles are cleared from the lung. You can sneeze or cough them out, and that would be from the upper respiratory tract. If they're in the areas where there is cilia, you can have cilia transport and they're ingested. They can be phagocytized by macrophages. They could be dissolved and be absorbed into your systemic system, or they could also enter the lymphatic system.

Metals are exposed to the skin, so there's a potential for dermal absorption for these elements or elemental compounds. Here's a picture of skin. Just remember that it has the epidermis, the dermis and the subcutis area. The epidermis has the stratum corneum,

which are dead cells filled with keratin.

Dermal absorption is a process of passive diffusion, and one of the determinants is the site of exposure. For example, the scalp is going to be more permeable than the palms of your hands. Chemical vehicle properties, something is ionized it's less likely to be absorbed through the skin.

Vehicle properties that can damage the skin or make the chemical more soluble. Skin age and health. Another determinant is species. Mouse skin is generally more permeable than human skin.

Quite a while ago I had a post-doc in the lab, Mohammad Rahman, and we were looking at the in vitro dermal absorption of a series of methylated arsenicals. This is MSMA, it's an herbicide. It was labeled here in this carbon group right here, C14. We used mouse skin in an in vitro system, and the 24-hour exposure using what's called the Bronaugh system. The day before the experiment, we cut the hair off the skin. The next day we sacrificed them, and we prepared skin discs. You just have a punch. And then you mount them in these Teflon diffusion cells and pump receptor fluid

below it. That's supposed to act like the systemic circulation.

You put the chemical on top of the skin and you wait 24 hours. The receptor fluid flows into scintillation vials ina fraction collector.

Then at 24 hours we wash the skin to remove unabsorbed chemical. We quantitate the radioactivity in the skin and what penetrated through. As you can see, most of the chemical was washed off from the skin, didn't penetrate. A small amount was in the skin. A little bit less was in the receptor fluid. But it did diffuse through the skin.

This is for GI absorption and internal. What I mean by "internal" is what occurs once it's in the systemic circulation, because it pretty much is the same thing. Well, first of all, there's passive diffusion, and the determinants are lipid solubility and ionization. Just remember that the -- people may think metals are always water soluble, but it's not because as I showed before with the -- there's methylated, there's alkylated, highly methylated metals. That's the one thing you should consider, or

remember. And you have these things call aqua(glycero)porins. This is a membrane through the -- a channel through the membrane, plasma membrane right here. They transport small uncharged solubles, water and glycerol, but these compounds -- like this would be arsenite and ionized. And antimony and silicon have similar structure with glycerol, so they're able to go through these aquaporins. This is from a study from Mandel, et al, and they isolated Leishmania cells from people who had been infected with them. This was in India. It's a protozoan. They found that some of these Leishmania protozoan are sensitive to antimony, it's a standard treatment, and some are resistant. What they found is that the resistant one, they didn't have the mRNA for the aquaporins.They transfected them with additional AQP1. As you can see the amount of antimony that goes into the cells increased. The resistant one doesn't have the mRNA for this channel. As you can see it has less antimony compared to the sensitive one. Then they transfected them with the AQP1 and the antimony amount increased by diffusing into the cells. I should say also these go both ways.

Some of these go in and some are out, as far as the transport.

This is the really fascinating part about the absorption of the metals, is there's these transporters. You have divalent cation transporters for metals like iron and manganese. A lot of these I guess you can call them promiscuous because there may be one for iron, but these other divalent cations can also be transported by these transporters. Some are proton symporters. You have zinc transporters, copper, ATP-driven transporters. Then you have endocytosis. This is when the plasma membrane encircles a metal, a particle and envelops it to make this vesicle that goes within a cell.

This is what's known aboutiron absorption into the enterocyte. Now, the absorption of iron is very highly regulated, because iron can go through the Fenton reaction, which forms hydroxy radical. I'm going to talk about that in a few minutes. But the body highly regulates how much iron is absorbed and also how it's stored within you.

Here's an enterocyte. This would be the

intestine and over here would be the systemic circulation. There are basically three types of iron that would be presented to the enterocyte, heme iron, ferritin, and the iron cation itself. Heme iron is absorbed through a folate transporter. Then hemoygenase can degrade it to ferrous iron. Iron is taken up by receptor-mediated endocytosis. And then somehow, it's not known, but it can be degraded to ferrous iron. Then for iron 3, it has to be reduced by a reductase first, and it's actually the ferric iron. This is a divalent metal cation transporter and along with hydrogen is taken up. Then you have this pool of ferrous iron. It could be stored as ferritin, or it can be transported out by this ferriportin transporter. It is hephaestin which oxidizes the ferrous iron to ferric form. Then this binds with apoferritin in the circulation to iron transferrin. Also iron can bind to small molecules. So if you see NTBI it is non-transferrin bound iron. That's what NTBI -- if you see that on medical reports, if you go to get a physical and they check your blood for iron.

Iron is distributed. Transferrin, a blood

glycoprotein, can bind several irons in the plus 3 oxidation state. It's primarily for iron, but it can bind these. Ferritin's a globular protein, and it's more of a storage form of iron. It stores a whole lot. It's kind of interesting how much iron can be stored. It's not like one or two molecules. It's really a whole lot. Albumin can bind divalent metals and metallothionein is an inducible cysteine rich protein, binding things like cadmium, zinc, and mercury.

Metals go to different sites within the body. For example, mercury vapor and methylmercury go to the brain. They cross the blood brain barrier, oxidize into the mercuric form. Monomethyl mercury is demethylated and oxidized to the same form. Lead goes to skeleton. Cadmium, kidney and liver. Arsenic goes to -- it likes sulfur groups, so distributes to the skin, nails and hair. If you're overexposed to arsenic or thorium, you can get these things called lease Mees' lines. It's also in the literature, –that Napoleon was murdered or poisoned with arsenic. he died in 1821.They found arsenic in his hair, but other people analyzed the hair too and they don't find

arsenic. So it's just a big question mark. It's kind of interesting how the death of Napoleon, there's a big argument about it. It's interesting about the history of arsenic, that's all. I should say that the coroner said he died from stomach ulcers, and ended up being stomach cancer.

The metals are metabolized. They can be reduced and this is non-enzymatic. For example, chromium 6 can be reduced to chromium 3 by ascorbic glutathione cystine. Arsenic can be reduced by -- 5-glutathiones to this tri-glutathione complex, and it goes from pentavalent to trivalent form.

Now, for humans, the reduction is not well characterized for metals. It's really characterized for bacteria -- well, that's where it's really characterized pretty well.

A colleague of mine at EPA, David Thomas, he's been looking at the metabolism of arsenicals by the intestinal microbes. They've been using mouse cecum. Now, I have a picture here of a human. I just was looking for a picture of intestine. So just remember, their study, they used mouse cecum.

But anyway, in the intestine we have a reducing environment, there's sulfur and methyl groups, and so they're finding all these sulfur compounds in their incubation. They don't know if these are absorbed or these are just eliminated. And there's really not a lot known about these, their toxic properties of these thiolated arsenicals.

Metals are methylated in the environment. Arsenic, bismuth, cadmium, et cetera. Some are even higher alkylated, like dimethylmercury. Lead is higher alkylated. What's known in the humans is the arsenic, bismuth and selenium are biomethylated. I think what's best characterized is the methylation of arsenic. About 90 years ago or so Frederick Challenger proposed a scheme called oxidative methylation, and he was working with a fungus, and they were able to characterize or isolate some metabolites, but not all. He came up with a scheme. His proposal is that if you start with arsenate, which is pentavalent arsenic, it's reduced by two electrons to arsenite, and then somehow it's methylated and then this is oxidized to this momo methyl intermediate which

is pentavalent. So you have oxidative methylation. Then this can be further reduced to a trivalent intermediate. Right here people talk about MMA3 and MMA5 at the meeting or DMA3 and DMA5, if you go to the arsenic sessions. This would be MMA5 and MMA3. MMA3 is methylated and oxidized at the same time to this dimethyl arsinic acid. This would be DMA5 reduced to DMA3,and methylated to trimethyl arsine oxide.

So the first reduction, it's not really known what happens within the human body or in vivo. There Hungary, and Vas Aposian, they found that in vitro, this polynucleotide phosphorylase can reduce arsenate to arsenite. And Zoltan Gregus has done further a lot of nice studies looking at this reduction.he's found that glyceraldehyde phosphate dehydrogenase is able to do this too, this reduction.

Now, my colleague at EPA, David Thomas, his laboratory found that this methylation is catalyzed by an enzyme called arsenic +3-methyltransferase, which is a very interesting enzyme. No one knows what its natural function is, but it catalyzes the methylation pretty well, and it's in a lot of different species,

but it also has a lot of different polymorphisms in that you can get different ratios of the methyl and dimethyl species.

So if you start with -- and it does have some reductive properties, because if you start with arsenite, you'll go through this whole pathway.

So AS3MT, starting with arsenate you do get methylation and reduction.

Now, pre-2000, it was considered this pathway, which still wasn't known, David found this, I think he reported it in 2001, that this was a detoxification mechanism. That's because these intermediates, -- this would be MMA3 and DMA3 were proposed intermediates. No one had detected them in any kind of biological fluid such as urine or even in vitro, because they were pretty unstable and the analytical techniques weren't available. They were able to detect these pentavalent, methyl and dimethyl species. And these are relatively much less toxic than arsenite. Arsenite is really very potent acutely, but then about -- a little bit before -- about 2000 there were reports, I think Vas Aposhian's lab with, I think it

was Chris Lee, they were able to detect MMA3 in the urine.Further studies show that this is almost as equally toxic as arsenite.

It suggested then that this is actually a -- this is a double edge sword, because you form these pentavalent forms that are excreted very quickly, but you also form these trivalent intermediates, these methylated intermediates which are fairly potent toxicants. And Sam Cohen's lab out in Nebraska has shown that this is a rat bladder carcinogen. This is the form that is carcinogenic in rat bladder.

And there's -- finally, there is an alternative pathway, and that's reductive methylation. That's because the trivalent arsenics can bind to sulfur groups in proteins. What would occur is that arsenite would be bind to a sulfur group, and that is then methylated, instead of going through this pathway of oxidation, reduction and methylation.

Then just to show you the importance of this enzyme, David developed a knockout mouse. It's a viable animal. We had radiolabelled arsenate and administered it to the mice and monitored them in a

whole body counter over a 96-hour period. The closed squares are the knockout mice, and the open squares are the wild type. So as you can see there's very little -- this is retention fraction of the initial dose, and here's time, and the mice eliminate the arsenic, the radioactivity really quickly, whereas these mice retain it.

Basically methylation does facilitate excretion, but you do form these trivalent methyl forms which are potentially pretty toxic .

The methylated metals can be demethylated. The intestinal microbes can demethylate methylmercury, so this is excreted. It can undergo enterohepatic circulation, but the microbes can demethylate it and forming inorganic mercury which is excreted in feces. The microflora in preweaned infants intestines is different, so they have less demethylation and they're susceptible to enterohepatic circulation with methylmercury.

Methylmercury can be demethylated, organolead. This is a CYP450 reaction. There's some evidence that, tetramethyl lead, the rate of demethylation is

greater than tetraethyl lead. And you form a variety of products.

Metals are eliminated in the urine. They go through the glomerulus and they're excreted. Some can be reabsorbed. For example, metallothionein cadmium goes through the glomerulus, but there's potential for this complex to be reabsorbed -- this is receptor-mediated endocytosis, proximal tubule; pH can affect the elimination of metals.

This complex here, this uranium bicarbonate complex, if you alkalize the urine, this is going to be excreted. If it's acidic, it breaks down and the uranium can bind the proximal tubule and damage it.

Metals are eliminated in the GI tract. You can get normal shedding of the enterocytes. For example, if they're taken up in the enterocytes and they're not absorbed systemically, they're just shed off. There's biliary elimination. For example, aluminum, arsenic, cadmium have been shown to be excreted in bile. For arsenic there's the MRP2 transporter. And this study by Kallah, et al, they administered sodium arsenite IV and they collected the

bile, and they were able to show that there's this arsenic tri-glutathione complex, and this model methyl arsenic di-glutathione complex are eliminated in the bile, whereas this one, this is a dimethyl, it's not. They didn't see it. And I talked about the methylmercury undergoing biliary elimination.

I'm going to say a few words about mechanisms and then Dr. Costa will talk about epigenetics later today.

This is called mimicry. This is when the metals look a lot like -- are very similar in structure, radius. Chemistry is somewhat similar. The transporters can be somewhat promiscuous; the iron transporter can also take up nickel, manganese, and cobalt. This is all divalent. Zinc transporter, several different metals, sulfate transporter, selenate, arsenate -- I'm sorry, phosphate would be arsenate.

So in this study here by Ganter and Idy, they transfected these erythroleukemic cells with the ZIP transporter, and they used radiolabeled zinc. And they included then -- so as you can see here, they have a

hundred percent with just the zinc alone. Then if they included in the cells these different divalent metals, you can see decrease in the zinc that's being uptake. These metals mimic zinc in the transport.

This one is called arsenolysis. You can see up here in the corner the arsenate has a very similar structure to phosphate. In this pathway, this is the 3 phosphoglyceraldehyde,this dehydrogenase can couple the phosphate here to this carbon to form 1, 3 diphosphoglycerate, and then the kinase can cleave this to form ATP. Arsenate has similar structure to phosphate. The enzyme can couple the arsenate to this glyceraldehyde, but you get just -- this isn't a very stable bond here, and water can hydrolyze it, so you can see you get loss of -- there's no ATP formed. This would be called arsenolysis.

Finally, LAT is the large amino acid transporter. As you can see here, methionine is an amino acid. It looks very similar in structure to methylmercury cystine.

This study here they looked at the oocytes that have been transfected with these transporters.

This LAT here, this box should be LAT1.

Anyway, you can see that the two different transporters take up the methionine at different rates and amounts. And this mimic right here, the methylmercury cystine can also be taken up too.

You'll see a lot in the literature on oxidative stress in metals. These are free radicals and reactive oxygen species, the nitrogen center species such as the peroxyl radical, hydroxyl radical, hydrogen peroxide. Then you have these nitrogen-centered radicals. Some of them are second messengers, so the cell is always checking its cellular oxidant state, because they always have enzymes and antioxidants such as glutathione; enzymes would be catalase.

If you increase the radicals within a cell or the oxygen or nitrogen species and/or decrease your antioxidants, you're going to be in a state of oxidative stress.Oxidative stress has a role in aging and human diseases such as cancer and cardiovascular disease. They have this famous reaction called the Fenton reaction. You have hydrogen peroxide and the

ferrous iron and it cleaves this to form a hydroxy radical, a hydroxylamine and the ferric form of iron. These metals can replace iron in the same reaction. Hydroxy radical is very reactive. You can form these other radicals here by different mechanisms. This can cause DNA damage, oxidized protein and lipid poor oxidation.

Metals are genotoxic. These are known human carcinogens: Arsenic, beryllium, cadmium, chromium and nickel. Most of them are not direct mutagens, but they are genotoxic. Micronuclei and chromosomal aberrations are some of the events that occur. Arsenic and cadmium, nickel and chromium, they can be -- are genotoxic through an oxidative stress mechanism. Chromium 6 is reduced and this can form DNA single strand breaks and this interesting complex right here.

Metals can also interfere with the signal pathways and transcription factors. Ones that are studied, arsenic is studied, well-studied, cadmium, chromium. And this study by Chen and Shaikh, they were looking at kidney cells and incubating them with cadmium chloride.At approximately 2.5 micromolar

concentration, they were able to detect reactive oxygen species within the cells. They saw this transcription factor, antioxidant response element binding and they were able to detect – an increase in the antioxidant enzymes.

At higher concentrations, though, they did see the reactive oxygen species increase, but these cells went apoptotic. So they couldn't overcome this higher concentration of cadmium chloride.

Some of the influencing factors, for example oral drugs, alcohol and tobacco.

Oral contraceptives can increase ceruloplasmin and increase serum copper. This is kind of interesting. Cobalt in beer, you get increased cardiomyopathy; age and sex, the gene environment. This is -- you might, if you go to the arsenic section a lot of people are talking about this. This is polymorphisms. And what people do is they will look at the urinary DMA and MMA ratio.They're finding with different polymorphisms, AS3MT, you get different ratios of these -- of these methylated species, and some of it's related to skin lesions. There's a

Mexican population that has been studied. And it was related to the urinary DMA, MMA ratio to these lesions. Metals can also interact with other metals.

Finally, chelation, that's a way to treat metals that are within us. Chele- is a Latin word for claw of a lobster. It undergoes coordination chemistry. Your chelator is going to decrease toxicity of the metal. It's going to be water soluble. It will penetrate membranes, distribute the same as the metal. It will compete with natural chelators. The metal-chelate complex is non-toxic and it's rapidly eliminated.

Here's a couple examples of chelators. These are all sulfur ones. It doesn't have to be a sulfur compound to be a chelator. But an example is the 2, 3-dimercaptopropane sulfonate and dimercaptosuccinic acid. This is one here called British Anti-Lewisite, and it's also 2, 3-dipropanol.

This is actually another interesting story. Lewisite is a war gas that was developed by the Americans at the end of World War I, but it wasn't used; When World War II was approaching, the British

were worried that the Germans would use this war gas. Because if you read about World War I, a lot of war gases were used.

So the British set out to find an antidote for this, and they came up with this compound called British Anti-Lewisite. And basically with the chelators that you form a -- for this one, you form a stable five member ring. This works pretty (well), this compound, with arsenic and some other metals.

So there are some benefits and risks to chelators. They're effective against acute poisoning. Their complex is -- well, should be non-toxic, you move the metals from the soft tissues. And if it's orally administrated, that would be great. There are some risks, though, at times, because a toxic metal can be redistributed to other tissues or organs. It could bind to essential metals, which would be not a good thing. Metals just remain within the intercellular sites and never eliminated. They could have some toxicity.

In conclusion then, we note chemical properties of metals are important determinants in

their disposition and toxicity. Metals are important components of molecules and essential to life. They're absorbed by all three routes: pulmonary, dermal, GI tract. They're metabolized by oxygenation reduction mechanisms, and they're methylated and can be demethylated. There are a number of toxicities for these compounds, as well as influencing factors. And chelators can result in metal detoxification.

And I'm done. So if you have any questions. Sir?

FROM THE AUDIENCE: I think in one of your slides, you talked about toxicity associated with deficiency. What's the difference between deficiency and toxicity as now people are talking about toxicity, but I mean, is deficiency really a toxicity?

DR. HUGHES: Well, I would -- I think in the -- I would think it would be. I mean, because you -- well, I mean there is the low dose, you were talking about these low dose effects that basically it's a mono -- this monotonic. The U-shaped curve is what it is. It's with -- Ed Calabrese is always publishing about. So, it's -- I mean, yeah, it is a

toxic state, but it has -- it's unusual from what -- it's not what we classically learn.

FROM THE AUDIENCE: Yeah.

DR. HUGHES: You know, we're always thinking about, you know, dose -- increase dose, increase toxicity. So, I mean, I would agree with you, yeah, it is a toxic state because you do have these toxicities, anemia and Keshon disease.

FROM THE AUDIENCE: Just another question. Talking about insoluble metals such as the carbonites, but we can still get toxicity with insoluble fractions, can't we?

DR. HUGHES: Well, the metal -- basically the -- for the most part they need to be soluble. I mean, you can get -- I would think you can get some -- I'm thinking of some type of reactivity on skin or something, like a metal particle or something gets on the skin. But I don't know how much that is soluble. But I think for the most part that things need to be -- metals need to be soluble, especially to be absorbed.

FROM THE AUDIENCE: We've seen lead carbonite being absorbed into the body in pollution events. I

don't know whether that's because it gets -- the form gets changed for it to be absorbed, because we can find lead in the blood at elevated levels, or just where it just overwhelms the system.

DR. HUGHES: Is this through GI absorption? I mean, GI tract?

FROM THE AUDIENCE: Yes.

DR. HUGHES: So, you know, it could be just a particle that goes through, like receptor mediated endocytosis or something like that perhaps. I mean, so maybe that's not really -- somehow it's the particles there, but it's not -- it's in the solution -- I mean, it's in your GI tract obviously, so it's in the liquid. It's there and it just comes across the enterocyte and the enterocyte somehow envelops it and takes it in.

FROM THE AUDIENCE: Thank you.

FROM THE AUDIENCE: Just a real quick question. You had your list of essential metals and you listed six with chromium as a maybe. I sort of had the impression there were a lot of others that were in play and discussion out there that might have essential functions but it's unproven or controversial. How

solid is that list? I mean, one that comes to mind that I've seen suggested is vanadium, and I think there are others as well, but I mean, it's really hard to create a deficiency, so it's kind of uncertain.

DR. HUGHES: Right. Right. From my read, those are the ones that come across all the time. I mean, so -- I mean -- but you're right, there's other metals that people say are essential. I've worked a lot with arsenic, and so at one point people are saying, well, is arsenic an essential metal? Because if you throw it into -- well, there's one report saying, I think it was -- I can't recall the person's name, but they were saying that, you know, arsenic is essential because in their studies with animals they took it out. But as you said, it's really hard to take out these metals from the diets.

So, to answer your question, as far as my reading is that those are the main ones. And so there are some types of metals that could be important for other enzymes, like you said, vanadium.

FROM THE AUDIENCE: Okay. Thank you.

FROM THE AUDIENCE: Hi. I had a question

about the (inaudible) phenomenon. Is that specific to water or do other solutions consisting of poly molecules also have this phenomenon where metals can dissolve more easily as opposed to in gas?

DR. HUGHES: Well, it's the -- the -- well in a gas there's nothing there that -- generally my read of it is that there's no -- that can decrease the energy of the ionization. Because you just have the other gaseous molecules there. So I would think that something that can form a dipole moment, because the water can form the -- you know the oxygen and withdraws the electrons from hydrogen, so it forms that dipole. So something -- another -- certainly another something in liquid state that can form a dipole should be able to do this. But it's -- for humans, it's not -- at least within us, you know, we're full of water. So that's going to be really the important thing for us. So I would think chemically, yes, there are other things that, for example, maybe chloroform. Chloroform could be like -- that could potentially do that too because you have the chlorines withdrawing the electrons from the carbon. So I'm just making the

suggestion there, but I'm not for sure.

FROM THE AUDIENCE: Thank you.

(Whereupon, the presentation was concluded.)

SOCIETY OF TOXICOLOGY

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Course: Toxic Effects of Metals

Presentation: Metal-Induced Organ Systems Toxicities

Speaker: Dr. Wei Zheng

DR. WAALKES: So it's my pleasure now to introduce our next speaker, Dr. Wei Zheng. And Dr. Zheng is a professor of Toxicology at Purdue University. He began his career at Columbia University in the School of Public Health, and he joined Purdue in 2003. Now he is the head of the School of Health Sciences. And there's a number of faculty and staff that he's in charge of. And his research interests are in the metal-induced neurodegenerative disorder, such as Parkinson's disease, Alzheimer's disease and essential tremors. And he has a special interest in manganese-induced Parkinsonism and lead-induce neuro deficits.

DR. ZHENG: Thank you, Mike, for your introduction. Good morning, everyone. This lecture is mainly about the metal toxic effect on the organ systems. There are three organ systems we're going to talk about, that is, brain, blood vessel, and bones. Also, we will use several well-known toxic metal such as manganese, lead, and aluminum to address those issues.

Now, what I'm going to do is that first I'd like to very briefly review the unique biochemical property of toxic metals. Then I'd like to discuss the metal interaction with the three B organ system. And finally, I'd like to take this opportunity to introduce some new technology in the metal toxicological research. I hope this lecture will provide some food for thoughts.

Now, if you look at your handouts, you will find that I've tried to put as much as information there, but I'm not going to go through each line. So even some lines I did not mention; that does not necessarily mean it's not important. They are going to be your take home message or your homework; okay? Also

some slides in the handouts came out not as ideal as I wanted. So here I apologize for this.

Now, as Mike Waalkes, the first speaker, just mentioned, metal is very unique in their special way. Unlike the organic chemicals, the metals may change their chemical forms; but their basic units are neither created nor destroyed; the lead is always lead and cadmium is always cadmium, no matter how human manipulated them. That's why the metal has a long persistent biological effect. They can damage one organ system; but if they are not sequestered, they can run to the other organ systems and cause the similar damage.

So from the toxicological point of view, many metals are intracellularly distributed. For example, the manganese is not only intracellularly distributed, it is also accumulating in the nuclei, making it difficult to remove them. That makes the treatment very difficult. And that's why a lot of metals have very long half-life. Cadmium, for example, has the half-life about 30 years in the human body, and lead about 20 years.

Many metals, as Mike just pointed out, are transported across the cell membrane by unique transporter systems. The DMT1 transports the iron, but it also transports manganese. And the CTR1 transported the copper. The zinc proteins not just transport the zinc, but also transport some other heavy metals as well. Because of this unique transport property, any genetic modulation or the defect on those transporters can cause profound consequences in body accumulation of metals accumulation in the body such as in the case of Menkes disease and Wilson's disease we will talk about in the next slide.

From neurotoxicology point of view, metals have unique distribution pattern in brain. Our recent study by synchrotron x-ray fluorescence technique has found that lead in the brain is relatively evenly distributed, but for copper and zinc, they are highly-concentrated in the hippocampus.

This slide shows a list of typical neurotoxic metals. Lead is well-known to induce the learning defects, but the most recent data from the human study and animal data show that lead exposure may contribute

to the etiology of Alzheimer's disease. On Tuesday afternoon, there will be a symposium specifically discussing this subject and Dr. Jennifer Freeman from Purdue University will chair that session.

We will talk about manganese in the next slide. As we just mentioned copper, it has a U-shape dose-response curve. When the concentration is low, it is useful but then the concentration is high, it can be toxic. Copper requires ATP7A to enter the brain. A genetic defect in ATP7A can cause significant copper defects. The brain function requires copper. That's why ATP7A deficiency causes Menke’s disease.

ATP7B, on the other hand, transports copper from liver to bile and actually remove the copper from the body, and also removes the copper from the brain to the blood. So the genetic disease that happen in this ATP7B eventually can lead to the copper overload in the brain. In clinics we call it the Wilson's disease.

Iron has been implicated in the Parkinson's disease. Early exposure to mercury can cause after births learning problems. We will talk about aluminum later in the lecture.

Now I'd like to spend a little bit of time to talk about manganese. Manganese exposure happens mainly in the occupational settings, where steel workers and welders are most frequently the victims of Mn exposure. This table summarizes the similarity and differences between manganese and Parkinson's disease. Both manganism and IPD patients share the common signs and symptoms, such as increased muscle tense that leads to dystonic posture. On the left side of the slide, you can see the typical dystonic posture. Okay? Also you'll see the significant decrease of the body movement. It's very typical in manganism patients, also called a cock gait. Both manganism and IPD patients have the tremor. But often or not, you find the resting tremor in the Parkinson's disease, whereas manganism patients show the action tremor. In addition, the Parkinson's patients respond to levodopa therapy pretty well. That does not happen to the manganism patients. The other method to distinguish manganism from IPD is to use the MRI to see if manganese in brain striatum. We will mention this later. In the manganism patients, you will find a high

signal in striatum, but it does not happen to the Parkinson's patients.

Several years ago I believe in 2005, we did a human study using MRI. The subjects were recruited from the ferroalloy industry where workers are manufacturing the manganese. The top one MRI imaging was obtained from a control subject. For the low-dose exposure, workers were not in the front line, but worked in the office of the same factory so they were also exposed to manganese. The bottom one is the manganese worker; being smelters, they worked in the front line of production. You can clearly see there's a very bright signal in the globus pallidus.

This next cartoon shows you where is the globus pallidus. Here is the globus pallidus, okay? But our recent study from animal model used synchrotron x-ray fluorescent technique, and we found that manganese is actually also accumulated in the substantia nigra. We know that substantia nigra has dopamine neurons. So the damage to the substantia -- that often happens in Parkinson's patients – may be one of the mechanisms of manganese toxicity.

This slide shows you how we did this study. The animals were treated with the manganese over the period of time and we took the brain and prepare for synchrotron study. The Synchrotron generates very high speed particles. The particles then bombard the tissue, and generate this imaging. Now, from this slide, the top one is control, the lower one is manganese treated animal. You can see manganese is labeled in the red-color; the green color is iron, you see a lot of iron here. Copper is purple. After manganese exposure, the manganese clearly accumulates in the substantia nigra.

This slide shows you where substantia nigra is. We think that site of manganese accumulation in the brain is not just substantia nigra by the MRI, but is also in a substantia nigra. The site of manganese accumulation often can be a site of manganese toxicity. The data from the John Hopkins indeed reported that in primates, manganese decrease dopamine release. And the other investigator from the Washington University in St. Louis used the PET scan to study fluorodopa uptake. They found the fluorodopa uptake was reduced in both

manganism and IPD pateints, but manganese-exposed welders had more significant decrease in the caudate nuclei here, whereas the typical Parkinson's patients had the fluorodopa significant decrease in putamen. So there is difference between IPD and manganism.

So coming back to this slide, we think that Based on MRI and synchrotron studies, we know Mn accumulates in both GP and substantia nigra. So there is a sound reason to speculate that Mn intoxication may affect neuronal pathways in those two regions to cause pallidal degeneration as well as nigrostriatal dopamine dysfunction. But a lot more studies need to be done to verify this hypothesis.

Cellular types that are affected by the manganese exposure include DA neurons, GABAergic neurons or microglial cells. A couple years ago Ronald Tjeken at Colorado State did a very nice study, they found manganese exposure in very early stage can increase neuron’s susceptibility to the inflammatory glial activation in adults. So this is a relatively new area of the study.

Now, once inside of the cells, where does Mn

accumulate? For many years people believe that manganese is mainly accumulate in the mitochondria. To demonstrate if that hypothesis is true, Dr. Kalia in my laboratory conducted in vitro experiments. Very surprisingly we found that most of Mn ions in brain cells are in nuclei. In the blood-brain barrier Z310 and RBE4 cells, more than 70% of Mn are in nuclei. The PC12 and N27 cells, those are the dopamine neurons in the brain, less than five percent of the manganese accumulates in the mitochondria. Okay. So the majority manganese actually accumulates in the nuclei. But our study does not necessarily mean that the mitochondria is not important; it just simply point out that after exposure, nuclei play a very important role in the subcellular distribution of Mn. This observation raises new question, why Mn accumulates in nuclei, what are the consequences. Those can be excellent research subjects of graduate students

For mechanism of cellular toxicity, some of the studies demonstrate that manganese may interact with essential elements and can also generate the oxidative stress. But today what I'm going to talk

about is the -- this isn't going to work -- okay, so today what I'm going to talk about is -- manganese interaction with Aconitase and also called iron regulatory protein-1. And this slide shows you that aconitase has four iron, four sulfur cluster in its active center, which is involved in ATP production in Crab circle. When the cellular iron concentration is low, the enzyme lose its forth Fe and become 3 iron four sulfur as iron regulatory protein-1 involved in iron regulation, okay? So this active center actually is interchangeable.

One of the key proteins in iron regulation is called transferrin reception or TfR, which transports Fe into the cells. The mRNA that encodes TfR has the unique 3’-iron responsible elements that can bind to IRP1. Okay? When the cell needs iron, the IRP1 binds to TfR mRNA; the binding eventually stabilizes the transferrin receptor and leads to the production of TfR. So the more the transferrin receptor on the cell surface, the more iron that can be taken up by the cells. So the cellular Fe level increases.

But in case of manganese exposure, manganese

replaces iron in the fourth binding site to stabilize this structure and stabilize the expression of TfR. As a consequence, the over-production of TfR leads the cell to accumulate excessive amounts of iron more than more than the cell really needs. We know that excess iron in the cells eventually can cause the oxidative stress and kill the cells. So this is what we think it may happen at the cellular level with Mn toxicity.

Okay. for the research challenges in the manganese-induced neurotoxicities, we think that early diagnosis of manganese intoxication is a key to the prevention. Mn parkinsonism is often found too late for therapy. Patients with the established signs and symptoms often do not respond to therapy. So the prevention is the key. Since Mn is intracellularly sequestered, it is a daunting task to find a reliable treatment for manganism.

From the Public Health point of view, manganism shares the similar symptoms with PD. Is it possible that Mn may sensitize some individuals making them more prone to the Parkinson’s disease? So, more epidemiological studies on human cohorts should be

done. We also don’t know What level in air is considered as safe. So the exposure assessment is still needed.

I’d like to point out a few New Trends in Mechanistic Investigation. We need to better understand early exposure to Mn and the toxic outcomes in later life. Since we have shown the evidence that Mn Acts on substantia nigra and DA pathways, more studies need to be done to prove this finding and seek for mechanism. Finally, Mn may not just interact with Fe; its interaction with other metal ions such as Cu, Zn should be investigated.

Now I'm going to shift the gear, talk a little bit about the metal toxicity on the vascular system.

This cartoon shows the typical vascular structure. The blood travels through the artery to small and narrow blood vessel called capillaries where the blood can exchange the materials with tissues. The remaining blood is eventually collected by the small venule and to the vein. The chemicals can cause the non-selective structural damage to blood vessles; okay? For example, the high level exposure to the cadmium or

lead can damage the endothelial cells and cause hemorrhage. Some metals can selectively cause the functional damage, for example, cadmium and lead can interact with blood vessel and cause the contraction of the blood vessel, in the clinic and one can see the hypertension. Manganese, on the other hand, can interact with blood vessel and dilate blood vessel, so in the clinic it causes the hypertension.

Some metals can be sequestered by the vascular system. For example, the choroid plexus, a tissue that has rich vascular structure, can accumulate more than 11 toxic metals. In the next few slides, I'd like to use lead as an example to show how toxic lead may damage vascular structure.

The experiment in this slide shows the direct effect of lead on the blood vessel. There is an extensive cerebral hemorrhage, that is due to lead exposure. If we use di-aminobenzidine to stain the red blood cells on the left, you see that in control without lead exposure the blood cells mainly stay inside of the blood vessel. But with the lead exposure, you will find the red blood cells actually

invade brain parenchyma, which suggests the leakage of the blood cells to brain tissues in the lead-exposed animals.

Several years ago, we used electron microscope, TEM, to study low level lead exposure on the blood-brain barrier. We used Lanthanum as a leakage marker because the molecule cannot penetrate blood vessel. So in the control group you will see all dark stains are confined within the blood vessel; but with the lead exposure chronically, at relatively low concentration you can clearly see the invation of lanthanum to brain parenchyma. So how can the lanthanum pass across the BBB? It must affect the permeability or the tight junctions that control the permeability; okay?

And we further did experiment to see what kind of tight junctions actually got damaged. We found that Pb exposure selectively reduces the expression of Claudin-1, but not occludin and ZO1. And the effect is more toward the membrane-bound claudin-1 than the cytosolic claudin-1. So the lead exposure indeed affects the tight junction.

And beyond this direct cytotoxic effect, some early studies have shown that Pb activates Protein kinase C, which in turn increases the permeability of the blood-brain barrier. This is a typical protein kinase C activity assay. We did a study years ago, I think, it was actually published in 1998. In our own experiments, we found that after lead treatment, PKC activity in the cytosol is decreased, but in the membrane, the PKC activity is actually significantly increased. The data suggest that PKC activity is activated after lead treatment. PKC activity is associated with the loss of endothelial barriers and also related to the increase transendothelial permeability.

So I'm not going to read also through all the lines on this slide, but one thing I'd like to point out here is that the blood brain barrier is very important in the etiology of neurodegenerative disorders particularly in Parkinson and Alzheimer’s diseases, because the blood-brain barrier actively transports the key proteins involving in the disease progression. Here I’d like to use beta-amyloid as an

example.

This slide just shows you that beta amyloid is a critical peptide involving in aggravation of amyloids, which is the key event in the formation of senile plaques in Alzheimer's disease. So the homeostasis of Abeta in brain extracellular fluids is critical to the progression of disease. So this slide shows you that blood brain barrier cells. The endothelial cells are connected by tight junctions; okay. Under normal condition, beta amyloid in the blood vessel can be transported into the brain by the protein called RAGE. Within the brain, the Abeta can be transported by the other protein called LRP-1 out of the brain back to the blood. So the influx of Abeta is mediated by RAGE and efflux is by LRP1. Okay. So these transporter systems at the blood brain barrier maintain the stability of the A-beta in the brain.

Several years ago, Dr. Janelle Crossgrove did the study to see if Pb exposure affects the Abeta clearance from the CSF. This is a choroid plexus, the tissue is in brain ventricles where the blood-cerebrospinal fluid barrier is located. Inside is the

blood vessel. Outside is the cerebral spinal fluid. As you can find, with lead treatment, you can see a lot of A-beta accumulate in epithelial cells. In other words, lead exposure increased A-beta accumulation in the choroid plexus. Okay. In other study, we also found lead exposure can activate protein kinase C, prompts LRP1 moving toward the apical membrane facing the CSF. Thus, Failure of the choroid plexus in removing Abeta from the CSF to blood results in Abeta build-up in the tissue. I will talk about all this in the symposium on the Tuesday afternoon. So if you want to learn more about it, I welcome you to attend that symposium.

Now, for the future research in this area, we think that recent epidemiological studies have established the relationship between lead exposure and Alzheimer's disease. Some altered genes related to amyloidal genesis have been associated with early lead exposure. We will have a special symposium on the Tuesday afternoon on this subject, so I'm not going to talk about it in great detail here.

And one thing I'd like to mention is that the

recent from our work shows that a fat-rich diet can increase the blood brain barrier permeability because the high fat diet contains abundant lipids, which can precipitate in the blood vessel and alter the permeability. It would be interesting to see if fat-rich diet enhances lead effect on the blood brain barrier permeability. How does all this have anything to do with the beta amyloid in the brain? So this is a very interesting research subject.

I'd like to mention that there is an increased interest to understand how the BBB transports the specific proteins in AD or PD such as Abeta and alpha-synuclein. Also there is a need to understand the signal transduction in BBB, such as LRRK2, and how that may affect metal transport. So there are a lot of research subjects for students to explore.

Now, I'm going to shift the gear to talk about lastly aspect about metal toxic effect on a skeleton system. This cartoon shows that structure of the bone. The bone actually has two major components, the cortical bone or the compact bone that is on the out surface. About 80 percent of the bone actually is

cortical bone. And also there are some spongy bones or the trabecular bone that is soft inside.

Our bone constantly experiences the circle of reabsorption and accretion. So those circles are very important for the metal exposure. When we talk about the metal exposure in bone, we often refer bone as the target organ for the metal toxic effect. Indeed, Mike just mentioned that cadmium accumulation in the bone can cause Itai-Itai disease; okay? The lead accumulation in the bone can soften bone and inhibit an osteoblast and sometime form the Pb line. But the bone is also a organ for the metal storage, okay? If you look at this table, you'll find out that 99.9 percent of calcium accumulate in the bone. For lead, on the other hand, 92 percent of the body burden of lead actually accumulates in the bone. For Aluminum about 34 percent in bone and Manganese in the human, 43 percent of manganese is accumulating in the bone.

The metal bone accumulation is kind of a defense mechanism for metal toxicity. When the metal gets into the body, the bone acts as a storage site to prevent metal toxicity from happening right away. But

before long, the bone actually can serve as internal source of exposure. Slow release of lead from the bone is a known source of chronic lead exposure.

Let's talk a little bit more about aluminum toxicity. The aluminum exposure happens in occupation and environmental settings, mainly is occupational; but more often than not, aluminum exposure happens in the clinical settings, or by the medication. For example, renal failure patients who receive the dialysis sometimes can develop the symptom called dialysis dementia. That is due to the aluminum present in the tubings or in the process we will mention as the parental nutrition in the next slides. Clinically, you will find out that aluminum exposure can cause the bone dystrophy and also soften bone. So some mechanistic studies have found out that aluminum can inhibit bone remodeling. In addition, aluminum itself can form the physical barrier to interfere with the bone calcification.

The aluminum osteotoxicity is quite often seen in parenteral feeding in infants. The infants through premature delivery, the pre-term infants, okay, often

receive the parenteral nutrition through those tubings. The process itself could contaminate by aluminum, because aluminum is ubiquitous and present everywhere. So the subjects or the infants by neonatal aluminum exposure often have low hip bone mass. That has been demonstrated in the clinic. If you want to know more about the detailed mechanism, I would invite you to read most recently review article about how the aluminum may cause the trouble.

Several months ago, we did a study to see if manganese exposure may lead to its accumulation in bone, what is the half-life and what is the time frame for manganese to build up in the bone. Dr. Lan Hong and Stephanie O'Neal a doctoral student, they are in the audience here, did the study. They treated animals with manganese. Over the period of time they killed the animals, took the bone samples and measured the manganese concentrations in the bone. This slide shows the results. You will find that the femur is there -- the femur and the tibia is really the leg bone, and the humerus is a hand bone, you will found out that over a period after chronic exposure manganese concentration

is indeed increased in the bone.

More importantly, their data found that there is a significant correlation between the manganese concentration in the bone and manganese concentration in the brain. In the striatum, for example, changes in manganese are the function of the manganese concentration in the bone. Same thing is true for the manganese concentration in cerebral spinal fluid. The change in the manganese in the spinal fluid is a function of the manganese concentration in the bone. Right now the study is still ongoing. We try to find out what is the half-life of manganese in the bone in those animals.

For the future study we think that the top challenge in metal osteotoxicity is how we can measure toxic metals in bone. Unlike blood, we cannot easily get bone samples for analysis. Thus, developing the non-invasive way to measure bone concentrations of the metals is critical. And there are new theories and practices in this area; I will mention this a bit later. I think we're very lucky at Purdue in that we have several outstanding researchers who are right now

doing this kind of methodology.

Also if you know the pharmacokinetics, the bone is a deep organ; a chemical or therapeutic drug is usually difficult to reach to that space. So treatment is actually challenging, how can we remove metals, in addition to brain, from the bone out of the body.

From the public house point of view measuring the body burden of the metal is always important for risk assessment. So there is a need to study if we can use bone metal concentrations as a new stand for occupational, environmental and nutritional monitoring of the trace metals.

I'd like to point out that a lot of new questions have never been answered in terms of the mechanistic understanding of the metal interactions with the bone, how the metals, for example, manganese, lead and aluminum, are accumulated in the bone, and what is the mechanism. Does the bone storage act as source of internal exposure. Many could argue that bone probably just simply stores metals and not necessarily releases them from the bone. But there are a lot open questions we don't know about. So I think

to the students in this room this is going to be a very good area for your study. The other questions such as the forms of the metals, how they are released from the bone, in what chemical forms, are they the same as the chemical form the human exposed to in the air, water and the soil, remain unknown. So a lot of study can be done in the field.

Now I'd like to use the last few minutes to talk about a new trend or new technology in the metal toxicological study. I think everybody in the room probably understand what is MRI, magnetic resonance imaging, and MRS, the S is for the spectroscopy. With the improvement of technology and reduced price, now MRI is used widely in nearly all mid-size hospitals. MRI is particularly useful for metal study because metals like manganese and iron in the high magnetic field can have a unique spin. So we can actually capture those signals, eventually generates the imaging. The advantages for the MRI are its noninvasive assessment, real-time measurement, and MRS even allows to see the functional changes. Any time physicians thought about manganese exposure, they can

always ask the patients to do MRI. This slide show the study we did in Nanning China for Mn exposure among smelters. You may recognize who is this big guinea pig. Dr. Ulrike Dydak is the leader in GABA analysis using MRS. So the MRI is not just to measure the metal concentration, but it also functionally tells you what kind of brain neurons is damaged.

The other technique I like to mention is called a Synchrotron base x-ray fluorescence imaging. The technique uniquely fits for metal study. The basic theory behind the x-ray imaging is that you inject the electrons into the system; the electrons can be accelerated at a very fast speed; so the high-speed particles can hit the target or bomb the target to cause the metals to produce the fluorescence. We can capture those fluorescent signals and then generate the imaging. The technique has now developed into the stage we can capture the signal from a single cell and we can see the imaging with single cells. The other advantage is that at the same time we can measure probably seven or eight different kinds of heavy metals.

This slide shows how that works. You have the synchrotron machine to generate high speed particles, the particles can be guided to the specimen mounted here, for example, the brain specimen or the liver specimen here. The particles hit the specimen and eventually generate fluorescence; once captured, you can see the pictures. This technique right now has become mature and has been widely used.

The other technique I like to mention is called a neutron-based x-ray fluorescence imaging. This technique is quite similar to the Synchrotron, but the source of the radiation is different. In this case, the source of radiation is the neutron. Now, that technique has advanced to the stage that you can build the neutron generator in a small volume so that it can be moveable if not portable. I think the portable neutron-base x-ray fluorescence technique is the future for bone metal assessment. That technique is going to change the field of metal study, metal toxicological investigation, and metal nutritional survey and monitoring. That is going to affect epidemiological study and risk assessment of metals in

humans. That is going to challenge the theories that guide our understanding of metals' health effects for so long time.

So in summary, I think that for the metal related to neurotoxic effect, new technology has allowed us now to precisely locate where the heavy metal is in the target area; especially with Synchrotron x-ray, we can get it down to the single cell level. But I think the most challenge issue to us in metal toxicology is really the metal toxicity to brain. We know lots of in vivo and in vitro studies have been done; but in many studies, up to this stage, the focus has been on cell's response to metal toxicity. The important thing is to integrate all the collected information in a systems way and to understand how metals interact with a particular or several neurological pathways. Because in the neuron -- - this is not happening in the single cell, it's actually the multiple factors come into play. So the systemic understanding what is the neurotoxic effect will be the future of mechanistic investigations. Also, I think that will be the basis for treatment in the

future.

Okay. For the metal interaction with vascular system, I think this area is under investigation. In the past, a lot of study have been done with biological understanding of the barriers and tight junctions, but so far not much has been done to understand the metal interaction with the vascular system. There are a lot of new opportunities for the graduate students.

Finally, about the bone, heavy metals are known to affect the bone. I think this area has been explored for many, many years. But recently re-energized interest is simply due to the technology advancement. Now, we have the opportunity to non-invasively measure those heavy metals in the bone. So I think this area offers a great hope and eventually the new opportunities for nearly all aspects of toxicological investigate in the future.

So I'd like to thank you very much for your attention.

Any questions?

FROM THE AUDIENCE: Yeah, we have a whole bunch of questions.

FROM THE AUDIENCE: I was interested in the XRF handheld analysis. We've done field work on environmental samples, soil and dust. Is that the same technology that you're using do the bone?

DR. ZHENG: Yes, we can use handheld device – it is the x-ray fluorescence device, for bone lead analysis. But the sensitivity may limit what you can do. If you talk about manganese, we primarily use MRI to detect manganese in the brain. It's not for the bone. For the bone, my colleagues at Purdue are developing a neutron-based device to detect bone manganese. I think it is the future of manganese investigation and risk assessment as I just mentioned. I really believe the neutron based x-ray is the direction for the future.

FROM THE AUDIENCE: Thank you.

FROM THE AUDIENCE: Okay. I was looking for the page. You made a very brief reference to arsenic affecting the vascular vessels. And I know that it cause angiosarcoma of the liver. Are you saying it causes increased vascular formation or decreased vascular formation, which?

DR. ZHENG: That actually is a very special area. I am not the expert in arsenic research but I know that arsenic can cause cancer. So somehow affect the androgenesis process. So I think properly the blood vessel is overproduced.

FROM THE AUDIENCE: Overproduced. That's what I would think. Thank you.

DR. ZHENG: Yes. Thank you.

DR. WAALKES: So we're scheduled for a coffee break at 10, so you have to stay here until 10. No, actually -- actually so come back please, here, at 10:30 and we'll reconvene with Max Costa.

SOCIETY OF TOXICOLOGY

MARCH 10-14, 2013

San Antonio, TX

Course: Toxic Effects of Metals

Presentation: Mode of Metals Toxicities: Example of Epigenetics

P R O C E E D I N G S

DR. WAALKES: Okay. So our next speaker is Dr. Max Costa. And he's held a number of positions throughout his career. He was at the University of Connecticut School of Medicine. He spent time here in Texas at Texas A&M, and at Texas Medical School and he's currently the professor chair at the Department of Environmental Medicine at New York University School of Medicine. And Max is well published in the areas of genotoxicity of metals. And today he's going to talk about epigenetics.

DR. COSTA: Thank you, and good morning. Welcome to San Antonio. We've never had an SOT meeting here, so this is the inauguration city. So I think

it's going to be good. It's small. You can't help but bump into each other, but anyway --

So, I guess one of the main objectives here is to get you to understand a little about the basic epigenetic machinery of the cell, DNA methylation, histone modification, micro RNA and gene expression, and to understand how metals affect this machinery to lead to diseases. Epigenetics is probably involved in every disease, but particularly in cancer and birth defects can be highlighted.

So I'm going to use some abbreviations. For histone modifications, I'll say histone H3K9 dime, dimethylation. And what I mean is down here for H3, histone H3, lysine 4 trimethylation, the K is lysine. I'll talk a lot about lysine because it's a site of extensive modification in the histones.

So, it's not always bad to review what you heard in the first lecture a little bit and reiterate some of the things that were mentioned. So, metals are transported a lot by similarities. So, iron, you know, binds to transferrin, and transferrin is also involved in the transport of these other metals, and in

particular, chromium 3 which looks like iron 3, which is bonded to transferrin. You heard about divalent metal transporter one, which is involved in the transport of iron and manganese, but it also can transport these other toxic metals such as nickel and cobalt. Cobalt is also essential. ZIP2 is involved in zinc transport, but manganese, cadmium and copper can be transported by ZIP2. Phosphate and sulfate mimicry. This is very important for hexavalent chromium, chromate, selenate, molybdate. You heard about organic metal complexes, the methylmercury cystine going in through the methionine transport system. Glutathione can be involved in transport of these metals. Calcium channels, cadmium and nickel can interact with calcium channels and block them.

So, in general, if you take an essential metal like calcium, you have these toxic metals that are actually quite toxic because of their interference with this essential metal. So, lead, for example, interferes with calcium. Calcium levels inside the cell are very low, they're micromolar concentrations, and about a thousand fold higher outside the cell, so

you don't need much lead inside the cell to interfere with intercellular calcium. Cadmium and other metals interfere with zinc. Nickel is very good at interfering with magnesium, and this is very important in the phosphate backbone of DNA and other magnesium-involving enzymes such as ATP using enzymes the nickel will interfere with magnesium. Iron, cobalt and nickel look just like iron in the periodic table. They're right next to each other. And so it's very easy for cobalt and nickel to affect iron. And copper, zinc, cadmium and molybdenum are known to interact with copper.

And metal binding proteins or molecules, metallothioneins we discussed, they are primarily there for copper, zinc homeostasis, but these metals can displace copper and zinc from metallothionein. Metallothionein can also bind these other heavy metals. Glutathione is very important in the detoxification of free radicals, but metals also bind to Glutathione and are involved in -- Glutathione is involved in some metal detoxification.

So, a bit of introduction to epigenetics. So,

at the level of DNA, you have one modification of DNA called 5-methylcytosine. It's often called the fifth base. And we know now that 5-methylcytosine can be metabolized by the TET protein to hydroxymethyl cytosine and eventually formal and carboxymethyl cytosine. And this is thought to be one way to burn off the methyl group from DNA. And methylation of DNA in the promoter region of genes turns off the gene expression, but methylation of thymidine in the coding region is very important for transcription. So if you have an exon that doesn't have DNA methylation on it, it won't be transcribed. So it's actually the opposite of what you see in promoter regions.

So this may be one of the mechanisms for alternate splicing. The exon has to be highly methylated in order to be transcribed. This is thought to be because the RNA polymerase needs to have a compact DNA in order to transcribe the DNA at the right position.

So only about one percent of the DNA is expressed in the genome, into RNA. The DNA in all of our tissues is identical. So within my body, all my

DNA is identical. My DNA differs from your DNA in single nucleotide polymorphisms, but what really makes my brain and liver what they are is the epigenetic program of gene expression. So it's epigenetics that really makes the tissue what it is. The brain is the brain and the liver is the liver because the epigenetics is the program of gene expression that's expressed from my DNA that makes those organs what they are.

So DNA methylation is one of the few modifications that we know that can control gene expression. And in relationship to cancer it's a recessive disease where many tumors suppressor genes are inactivated by DNA methylation of their promoters. And cancer is in fact a recessive disease. You can prove this by fusing a normal cell with a cancer cell, and when you do these kind of experiments, you find out that the resulting cell becomes the normal cell. So in general, cancer cells are missing genetic information, at least by these experiments.

So, what is epigenetics? What is the definition? It literally means “above genetics.” And

it was coined by Conrad Waddington. He had the first -- he was actually the first person to call this epigenetics. So epigenetics is defined as any inheritable influence on gene expression that is not accompanied by a change in the DNA sequence; okay? So a change in the DNA sequence would be a mutation, but a change in gene expression or a change in inherited function of DNA without a change in DNA sequence would be called epigenetics.

So Conrad Waddington was a developmental biologist and he was very interested in how cells developed, and so he had this -- those models that he made where he thought about what would happen if a cell migrated in different paths. And then he noticed that as the cell migrated this way, it assumed the -- the gene expression patterns of his neighboring cells here. But if it migrated this way, it assumed the gene expression patterns of the cells located over here. So whatever way the cell went, it would acquire its environmentally-related or nearby gene expression pattern. And he coined this phenomenon during development as epigenetics.

So as I said, the five position cytosine is methylated in most eukaryotic cells and all mammalian cells. Yeast cells are an exception. They don't methylate the cytosines. Bacteria methylate adenine, not cytosines, but that has to do with DNA replication and repair. It doesn't have to do with gene expression. So about five percent of all cytosines in DNA are methylated. And then methylation of cytosines in the promoter region turns off gene expression. And remember what I told you about exons, methylation of exons allows them to be transcribed. Exon is the coding region of a gene. It's methylated, it's transcribed. If it's not methylated, it tends not to be transcribed.

So this is very new information that's just coming out in the field. Actually they didn't know this at the time I made these slides, so --

So DNA methylation patterns are inherited. So, when the DNA replicates, the parental strand is methylated and the daughter strand is not methylated. Following DNA replication, a DNA maintenance methylation enzyme replicates the cytosine methylation

pattern of the parental strand onto the daughter strands. And this is called -- this is done by the maintenance methylation DMT1, but DMT1 also can do other things like de novo methylation.

Cytosine is found mostly in CpG islands where it's present once in every 15 dinucleotides, but in 98 percent of the genome is found once in every 80 nucleotides. And again, the source for methylation of cytosine is S-adenosylmethionine which is very important. Particularly you'll hear about it in arsenic metabolism and other -- all methylation reactions involve S-adenosylmethionine.

So the enzymes involved in DNA methylation, Dnmt1, first discovered enzyme by Timothy Bester at Columbia was the first to clone this. It's the maintenance methylation enzyme, but it also can do other things. This is not very important. Dnmt3a and 3b are the two de novo methylating enzymes. They're required for imprinting. This one is important in imprinting and to keep retrotransposons and other genes that shouldn't be expressed in the cell, they get highly methylated so that they don't move around and

they don't get expressed.

Now you should know that almost all of the DNA in our body is transcribed, okay? So this is contrary to what you read in books which say, okay, only genes are -- only a small percentage of the DNA is actually transcribed. In fact, all of the DNA is transcribed into not only genes which are a small percentage of the transcription, but non-coding RNAs which include micro RNAs, which interfere with gene expression, and other non-coding RNAs that regulate gene expression. So the methylation of the promoter regions of non-coding RNAs and micro RNAs is another way to stop the transcription of these non-coding entities within our genome.

So, very important methylation reaction of S-adenosylmethionine, adding methyl groups to DNA through the enzymes, methyltransferases. And so you always hear about the importance of folate in maintaining your methylation. And of course, that's because of this metabolism here, 5-methyltetrohydrofolate donates to the methyl group to make methionine, and then methionine is made into S-adenosylmethionines. So to regenerate

S-adenosylmethionine levels, you need folate. You need dietary folate. And it's very hard to get folate. It's just not present in foods. So of course, women who get pregnant, they put them on a folate-rich diet because it's hard to get folate concentrations. In Bangladesh, where people are exposed to high levels of arsenic, there's a lot of studies going on with folate supplementation because methylation of arsenic increases the excretion of the arsenic. So the more you methylate it, the more you can excrete it.

So you'll hear a lot of studies about the fact that S-adenosylmethonine can be depleted by arsenic, for example. And certainly chronic exposure can weigh in on S-adenosylmethionine, but the concentration of S-adenosylmethionine in the cell is about 70 micromolar, and it has a huge capacity to regenerate itself, whereas the arsenic concentration, if you have anywhere near a tenth to .5 micromolar in the cell, that's very high. So the numbers -- if you do the numbers, you find it very hard to think that S-adenosylmethionine depletion is involved in arsenic toxicity; however, you'll hear a lot of people say

this, and I think it's still very much up for discussion.

So, what controls DNA methylation? So, we go to the histones now, which are another tier of epigenetics. And the histone tails which protrude out of the nucleosomes are modified by various covalent modifications, such as phosphorylation, methylation, ubiquitination and acetylation, and all of these modifications are important in regulation gene expression.

So for example, H3 and H4 lysines are subject to acetylation and methylation. In general, any acetylation will neutralize the positive charge in the lysine and lead to the opening up of the nucleosome and greater transcription in the promoter region of genes. Now again, going to the coding region of genes, it's quite different now.

There are epigenetic modifications of histones in the coding region that tend to be silencing modifications. Again, similar to the DNA methylation that's required for the transcription of those genes. So it's a little bit different -- it's actually

opposite. When you think of the promoter region, the acetylation opening up chromatin and leading to condensed chromatin, the coding region you have more -- the coding region, you have more condensed chromatin and greater transcription.

So this is just a picture. These are nucleosomes. You can actually take a picture of these with atomic force microscopy, and you can see that in uncondensed nucleosome, they look like beads on a string. These are individual nucleosomes with the linker region. It's 142 bases of DNA wrapped around a histone octamer. And then a small linker region of DNA between the nucleosomes, so you have this cartoon is depicting what you can actually see when you look at nucleosomes from cells.

And the important feature here is that the tails, the end terminal tails of the histone molecules are protruding from the nucleosome. And these tails are subject to covalent modification. And the -- the enzymes that do these modifications are called writers, because they're writing what's called a histone code. So this -- so this is -- this histone code is then read

by other proteins that bind to it, that are called readers, and then the enzymes that take off the modifications are of course called erasers. So this whole -- Dr. Alice at Rockefeller was the first to propose this histone code hypothesis which seems to be very true.

So, the lysines on the histone tail, and let's just look at H3 here, you can see there's a lysine 9 here that's very important. And if this lysine 9 becomes acetylated, that's an activating modification in the promoter region of genes. The lysines are also subjected to methylation. They can be monodie or trimethylated. So dimethylated H3 lysine 9 is a silencing mark in the promoter of genes that are in euchromatin. If you get H3K9 trimethylation, that's a mark for heterochromatin, which is basically not expressed. It's highly condensed chromatin.

So these modification -- and the reason for this is very simple, that these modifications on the lysine attract other proteins. So for example H3K9 trimethylation will attract heterochromatin binding protein one which binds to it and causes the

condensation of the nucleosome. So those are the readers and the enzymes that modify them are the writers, and then there are enzymes that take them off that are the erasers. So all this, just to continue the serines in the histone tail are subject to phosphorylation. And the interesting serine here, H3 serine 10, and H3 is phosphorylated. This is thought to be a marker only of mitosis. So this happens a lot during the division of cells and the mitotic division of cells. This modification becomes prevalent and all of the H3s become phosphorylated. The phosphorylation of this of course interferes with the neighboring acetylation and methylation of this one. So this is very complicated. And these neighboring effects are very, very important and we're only beginning to understand them. And histones are mono ubiquitinated, and the carboxyline, you have H2A and H2B that are ubiquitinated, and those are also involved in gene expression and other gene activation or gene silencing.

And so, if you wanted to depict a nucleosome where the DNA is shown here -- and this nucleosome all the lysines are acetylated. The nucleosome is

decondensed, and the DNA has access to transcription factors to bind it to activate gene expression. So this is what happens when you have acetylation of lysines and lack of methylation of H3K9 or H3K27, which are silencing marks.

And then in the right, you have a highly-condensed nucleosome where the lysines are not acetylated and the histones are highly condensed with the DNA, and the DNA does not have access to transcription factors so this promoter with this kind of nucleosome configuration will not be expressed. The genes will not be expressed. But you have to imagine now the coding region is more like this being expressed. So it's a very complicated system. And you know this just shows you, you know, a better picture of all of the modifications that we know of. And a lot of us in the field play around with H3, because H3 is very important, because you can see all of the modifications that occur in H3. And H3 is also located in the critical positions within the nucleosome, and so, it's a very important histone for modification, as is H4 too, but you can see H3 is actually modified a lot

more.

Okay so, the real question to ask yourself is, we know how DNA methylation can be inherited, because there's the DNA replicates and there's a maintenance methylation that recognizes a 5-methylcytosine in the parental strand and puts one in the daughter strand. Okay, so it's very simple. It works. And the DNA methylation pattern is replicated, and therefore, a change in DNA methylation is inherited in all subsequent cell generation.

However, histone modifications are also known to be inherited. So how does that happen? And this is a model that Dr. Moazaed proposed, which a lot of people now believe in. And so let's just go through this model. So this is the replication of DNA, and the nucleosomes with modifications, the histone modifications are placed in the parental and daughter stands randomly, okay? So here you have the nucleosomes in the parental and then the daughter strand. And what happens is a naive virgin nucleosome is deposited where there's a space for it next to the nucleus -- next to the nucleosome that displays during

DNA replication. So here is a new nucleosome with no modification on it. And the hypothesis here is that because of the neighboring modification that occurs, this modification is simply copied in the neighboring nucleosome that's a virgin, okay? So this virgin modification simply assumes the histone modification of its neighbor. And viola, that's how these histone modifications are inherited.

So obviously there's more work that needs to be done, but this offers an explanation of how histone modifications can be inherited from one generation to the next.

So let's talk about some metals. So, I've been working on nickel compounds since the late '70s trying to study nickel carcinogenesis, the reason being that occupational exposures to nickel in the nickel refining industry occurs a lot. So -- but we don't have any more nickel refineries in the United States because there's been so many litigations and lung cancers and nasal cancers that they've all been closed. However, it's very surprising to me that modern countries such as Canada and England, Siberia Canada,

for example -- one of the interesting things you should know, and probably this is going to be something you've never heard of, but all of the nickel that's mined in the world comes from meteors, okay? So, so in Siberia Canada they have a huge amount of nickel deposit there, but it's from a huge meteor that hit the Earth many millions of years ago, and they're just mining the nickel from the meteor. So all the nickel you have is from outer space, not from the Earth. So that's kind of an interesting story.

So Canada is considered a modern country, and they refine nickel and they still -- I mean the exposures are less than they used to be, but they're still getting exposed to carcinogenic nickel compounds. And, you know, you have nickel refineries in Wales and in Kristiansand, Norway and Finland. Russia has a city that's actually called Nickel. So if you wonder what they're doing, they're refining nickel, and it's a huge complex in the Kola peninsula way in the north of Russia. And China has a huge nickel refinery too. So the United States doesn't, but a lot of modern European countries still do refine nickel, because it's very

important in stainless steel production, building aircraft engines and other things.

And one of the major problems with nickel not only is cancer, but is contact dermatitis, which is a lot of people are allergic to nickel. About 20 percent of contact dermatitis is due to nickel allergies.

So environmental exposures to nickel come from oil and coal-burning power plants, particularly oil. For some reason, oil has nickel and vanadium in it. And I don't understand -- you know, oil comes from, you know, biological material, they say some of it's dinosaurs, but maybe plants mostly; and why oil has only nickel and vanadium in it to high concentrations is not known. But whenever you burn oil, you always find nickel and vanadium as the marker of oil. Coal has every element in the periodic, all the metals, so you get mercury, cadmium and other metals, but you also get nickel. So nickel is used for enzymes and bacteria in plants, urease, the helicobacter pylori which is the bacteria that causes stomach cancer has a urease that requires nickel for activity, it breaks down uria, forms nitrogen that neutralizes the acid and allows the

bacteria to colonize the stomach, bacteria dehydratase, but there is no known function for nickel in mammalian cells.

And the real danger with nickel is not the soluble nickel compounds, because in fact if the NTP did a study of nickel subsulfide and soluble nickel, and the soluble nickel did not produce lung cancers, but the nickel subsulfide, the insoluable forms of nickel, which is what the nickel refinery workers are exposed to, this is the map that they use for nickel refining, and the exposure to these is what's causing lung cancer and the very rare nasal cancer, which is prevalent in nickel refinery workers. So nickel refinery workers have a very high incidence of this extremely rare nasal cancer.

So, for many years, when I first started working on nickel carcinogenesis, I could never see any mutations that were induced by this chemical. And at that time, the whole carcinogenesis field was dominated by people who were looking for mutation. So I would get a review of my grant and they would say, where's the mutation? And I said to them, it's not there.

Sorry. I don't have a mutation to show you. But so then it must not be a carcinogen. But nickel subsulfide is such a good carcinogen compared to anything else, so can you imagine if you put this anywhere in an experimental animal, you get a hundred percent incidence of cancer at the site of administration. So I don't know of anything else -- it doesn't have to be metabolized, and it's very broad-acting, and it can even cause cancer in animal species such as the amphibian that does not get cancer from other carcinogens. So this is an amazing carcinogen to work with. And one of the reasons I was interested in studying it, is it's so simple, there's no metabolism, yet it's so broad-acting and so potent of a carcinogen. And soluble nickel ions, which are generated in the cell after the uptake of these particles are not very toxic. So this is important for its carcinogenic effects because it doesn't kill the cell, but it allows the cells to survive and epigenetic alterations occur inside the cell that can lead to cancer without killing the cell.

So this is the model for the lung epithelial

cells of the worker or the nasal cells that get exposed to these insoluable nickel compounds. And I call this -- this is like an epithelial cell. So this is not a macrophage. A macrophage is a professional phagocytosis cell; okay? It does it for a living. These cells do not phagocytize normally. They don't want to phagocytize things, but they will phagocytize these crystalline nickel subsulfide particles. So I call them phagotative phagocytes. These are non-professional phagocytosis.

And what happens is these crystalline nickel sulfide particles with the negative charge -- if they have a positive charge, they don't go into the cell -- but if they have the negative charge, they get phagocytized, they get contained in vacules, and inside the vacule, the nickel dissolves and produces very high concentrations of nickel inside the cell. So this is one of the reasons that you hear particulate air pollution is so bad as well, because the particles can attack one cell and produce very high concentrations of heavy metals and other things into the cell. And again, going back to the soluble nickel, goes in by

DMT1, but you cannot reach the kind of concentrations you do with these particles in the cell.

So this just gives you an appreciation of the kind of concentrations you can get if a cell eats one of these particles. So 1.45 micro particle will produce a nickel concentration of 250 millimolar if it should totally dissolve. A 4 micro particle will produce 4.75 molar. So you can see that just a small amount of dissolution of these particles inside the cell will give you a tremendous concentration of nickel in the cell, which of course is going to be the reason that these things are so carcinogenic. They're interacting with individual cells.

Okay. So, some data on epigenetics. So, if you treat cells -- these are human -- human lung cells. But you can do many different kinds of cells. With nickel you can find that there are changes in H3K9 dimethylation. The global levels of this increase, and this is doing a Western blot with specific antibodies to these modifications. And you can see this increases with time and with all the different cell types.

And we did not know the reason for this at the

time we were doing the studies; however, in 2006, Yi Zhang and Yang Zhu (ph) cloned the first oxidated histone demethylase, which is an enzyme that can actually remove lysine modifications on histone. So previous to this, to 2006, everybody in the epigenetics field said that histone methylation was irreversible. Very famous scientists would go around giving lectures saying it was irreversible; okay? So now there's an enzyme that will attack the H3K9 demethylation. It's a member of the dioxygenase superfamily of enzymes. These enzymes are the only reason you need ascorbic acid in your body. And we have to take it in by our diet. Rats and mice can make this, but we need to take it in from our diet. And ascorbic acid is only used for the dioxygenase enzyme.

So these enzymes have iron in an active site, and the iron with alpha ketoglutarate as a co-factor that gets decarboxylated attacks the methyl group here and oxydatively demethylates it to formaldehyde.

So for those of you who wonder about formaldehyde, there are tons of formaldehyde that are formed in your nucleus all the time. Thank God for

glutathione which is supposed to react with this and get rid of it, but it also attacks protein. So this is a whole nother area. The lysines and proteins get attacked by formaldehyde, and they get formulated. So there's a lot of formaldehyde formed in your nucleus when there's these oxidative demethylations.

So this enzyme is exquisitely sensitive to nickel. The binding constant of nickel for the active site of this enzyme is three times higher than iron. Nickel easily displaces iron from the active site of the enzyme, and then activates it. And so that's why you get accumulation of these marks, because the cell cannot take them off. And so we purify this enzyme by expression and baculovirus and insect cells. It's a large molecular weight protein. And so you need to use insect cells to express it. And then we added nickel, and you can see increasing concentrations of nickel inhibit at very low concentration. The IC50 is about 20, 25 micromolar. Inhibits the enzyme. And we've done a lot of structural studies using the histone demethylases and also AVH2, which is a DNA demethylase enzyme, to show what happens here is that you have iron

in the active site, iron is bound in the active site to two histidines and the carboxylic acid facial triad in what's called a jumonji domain. And then nickel comes in, displaces the iron, and now nickel is there. And when nickel is there, it's hexacoordinated, there's no room for oxygen to bind, the enzyme is inactive, and it cannot take off these demethylation of H3K9 and other marks, H3K4, trimethylation, H3K27, all of these marks require oxidative demethylases, and all of these are inhibited by nickel, as they would be inhibited by low oxygen tension, because the enzyme requires oxygen. They would be affected if you don't have alpha ketoglutarate, which you need from the mitochondria. So there are a lot of -- and if you produce oxidative stress, you oxidize as iron.

So these enzymes are subject to many, many environmental (inaudible), oxydative stress, depletion of ascorbic acid, mitochondrial dysfunction, low oxygen tension and exposure to nickel and cobalt, because both nickel and cobalt will easily displace this enzyme from its active site.

And so the ultimate experiment to prove this

is shown here. And what we did is we took a wild type ABH2, this is a dioxygenase, and made a mutation where we changed one of the histidines to alanine in the active site, and then we expressed the flag-tagged protein in cells, and then added -- after expressing the protein, we added radioactive nickel 63. And the wild-type enzyme bound it where it had no mutation. But if you mutated one of the active iron-binding sites, it lost the binding of nickel in the cell. So this proves that nickel can go in and really displace the iron in an intact cell to inhibit the enzyme.

Arsenic compounds, you heard a lot about arsenic, which is not a metal, it's a metalloid. So, anthropogenic sources are shown here. Arsenic is used in the pesticide industry. In fact, you've probably heard about the arsenic that's present in rice now, in particular the sugar from rice that's used in baby food. And you say, “Oh, that happens when the rice is grown in Bangladesh or China.” But, no, it's rice grown in the United States that's grown in cotton fields. And cotton fields, they use arsenic as pesticides for the cotton, which was fine, you don't

eat it. But now we're growing the rice in the cotton fields, and we're eating rice in America that has arsenic contamination from the pesticides that were used in the cotton field.

And then there's a good side of arsenic. Arsenic trioxide is very effective in the treatment of acute premyelocytic leukemia. It induces apoptosis differentiation and completely eradicates this disease.

So 150 million people in 70 countries are exposed to arsenic-contaminated drinking water. In Asia, 60 million people, about 35 million in Bangladesh. In the United States, though, it's not a trivial issue. Almost everyone who has their own well in the United States is going to get some arsenic exposure, because it's present pretty much everywhere, and they don't monitor the wells as they do for city water. So there's about 600,000 U.S. residents that are exposed to arsenic in wells at -- above the standards. The United States, the drinking water is ten parts per billion. In Bangladesh, it's still 50 parts per billion. So this just shows you that it's present now in any -- any sugar from rice has a lot of

arsenic in it.

So arsenic is a group one known human carcinogen. Exposure also causes cardiovascular disease, neurological defects, neurodevelopment deficits, diabetes and hypertension. So in addition to being a carcinogen causing lung cancer, bladder cancer, liver cancer, and other kinds of cancer -- in fact, I'm going to tell you about arsenic and chromium 6 are the only two chemical agents that if you drink them, you can get lung cancer, okay? Human studies have shown drinking arsenic and chromium 6 will produce lung cancer. I don't know of any other chemical like that. So that's a very interesting observation about these two metals.

So, oxidative stress and epigenetic disregulation are likely to be involved in arsenic adverse effects. The mechanism of arsenic oxidative stress, there are epigenetic effects, effects on cell proliferation, antioxidant response element, NRF2 and disruption of poly ADP-ribosylation which is disruption of DNA repair. And arsenic is able to induce global changes in histone modifications. It alters DNA

methylation patterns and it alters gene expression. So arsenic has a huge effect on the epigenome.

And again, you heard a lot about arsenic metabolism. Basically, if you can methylate arsenic, you can get rid of it faster. People who methylate arsenic do much better. So if you give folate, you can methylate it better. If you have genetic polymorphisms in the arsenic methyltransferase, you can get rid of the arsenic better than people who can't methylate it. And this is counterintuitive to every other metal, organic -- any organic metal complex is much more toxic than the non-organic. You can go methylmercury, tetraethyl lead, it doesn't matter. Here, though, it's been mystifying how an organic metal complex can be excreted more rapidly. It probably has to do with arsenic binding to proteins, and when it gets methylated, it doesn't bind to proteins as well. So it gets excreted more rapidly.

And a few words about chromium 6, which is also -- this is a mutagen and a carcinogen, but it also -- just because something is a mutagen and a carcinogen -- and believe me, the most potent mutagens

and carcinogens are the ones that have the biggest epigenetic effects. So don't get bogged down in this idea that something is either epigenetics or it's genotoxic. If it's genotoxic, it has better epigenetic effects than it has in genotoxicity. And chromium 6 is a good example of that. Chromium is found in welding. Chromium mine tailings. There's the famous Erin Brockovich case, Julia Roberts, you saw her picture. The two forms, chromium 3, is non-toxic. Chromium 6 is toxic. Chromium 3 is really not essential. You should probably call it a nutritional supplement. There's no known essentiality for it. Chromium 6 looks like phosphate. It's been called a trojan horse. It's actively taken up by the place of phosphate, gets everywhere in the body. Basically can cause cancer in many different cells types. Whereas chromium 3 is not taken up, and it's not toxic.

So, toxicity of chromium: Contact dermatitis, nosebleeds, liver damage, resembling like phosphate. Cancer, now more and more epidemiological studies are showing that chromium six can cause cancer wherever you get exposed to it. And the mechanism is the uptake of

the chromium 6 by the phosphate sulfate transporter. It interacts with DNA, produces DNA protein crosslinks, DNA damage. But it also induces epigenetic effects. It silences many genes such as mismatch repair. Almost all tumors induced by chromium do not have mismatch repair. And so it induces epigenetic silencing. And you can see by studies that it increases these histone marks similar to nickel, but by a different mechanism. Chromium 6 depletes ascorbic acid, because ascorbic acid is required to reduce chromium 6 to chromium 3. And the loss of ascorbic acid inhibits the dioxygenases which requires ascorbic acid for activity.

So, simple conclusion. The extent of the epigenetic effects of metals is only starting to be realized. And there's much more work to be done. And so this is a very important area where more and more research needs to be done. It's a relatively new area. And a lot of people who are getting into it are finding rather amazing effects of the most potent mutagenic genotoxic agent being the most potent epigenetic agent. So I thank you for your time.

DR. WAALKES: We have time for one quick

question. We can do this after. Will you be able to --

DR. COSTA: Sure.

DR. WAALKES: Afterwards, too? And before we -- any questions? So Max will be available for after.

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Course: Toxic Effects of Metals

Presentation: Metals in Carcinogenesis and Developmental Origins of Adult Disorders

Speaker: Dr. Erik Tokar

P R O C E E D I N G S

Dr. Hughes: So our next speaker is Erik Tokar. And Erik spent some time -- or went to the graduate program at Michigan State University. And then he went to the National Institute of Environmental Health Sciences Laboratory to do a postdoctoral fellowship with Mike Waalkes, and now he's continuing on as he's currently a biologist there in that same group, and actually they switched the National Toxicology Program. Erik is going to talk about metals and cancer today. Erik.

DR. TOKAR: Okay. Thanks, Mike. Thank you for being here, everybody. As Mike said, we'll be discussing metals and carcinogenesis and the developmental origins of adult disorders.

So you see my abbreviations that you'll see throughout the talk. Quickly, to go over the outline, the intro, I'll go over the multistep process of carcinogenesis which is very important during cancer formation. And I'll mention some of the hallmarks of cancer that are acquired during this process.

I'll talk about metals as carcinogens, their properties, why they are important, what makes them unique. We'll get into the carcinogenic metals, how they're defined. Focus mainly on the known human carcinogens, but I'll also mention some probable and possible human carcinogens. I'll discuss some mechanisms, and then we'll jump into stem cells. I don't think you can have a cancer talk without talking about stem cells, and also since we're talking about developmental origins of adult disorders, I think stem cells are very important in this process too.

Then we'll get into the developmental origins

of adult disorders, the Barker hypothesis which sort of started and brought attention to this whole field. And I'll discuss some examples of cancer and other disorders in rodents and humans and possible role of stem cells in this.

So carcinogenesis, as I said, is a multistep process. It starts with the initiation step. This is generally a mutation that usually inactivates or it's a mutation in a growth regulatory gene, usually a tumor suppressor or an oncogene, this leads to a rapidly proliferating cell type, and you get some abnormal growth.

Next step is promotion. You have further mutations. This leads to an actively proliferating tumor cell population, not necessarily malignant at this point, but it is now considered a tumor population; and further mutations in things such as DNA repair genes, more oncogenes, more tumor suppressors. Then it's in the progression stage. And then the critical steps are dissemination, invasion and metastasis. These are very critical as they are defining characteristics of malignant tumors that lead

to most of the deaths from cancer.

So during this process, cells acquire many characteristics. And in 2000 Hanahan and Weinberg published their famous paper in Cell, defining the classic biological properties of malignant cells, which they termed the hallmarks of cancer. And you can see them all listed here. Many of them have to do with growth regulation, resisting cell death, cells become resistant to apoptosis, and all the others you see running down from about 12:00 down to about 5:00 there, those all have to do with some sort of regulation of cell growth or disregulation of cell growth.

Already talked about invasion and metastasis and their importance in this. Induction of angiogenesis. This really feeds the tumor beyond what tissues would get through passive diffusion of nutrients. And important ones here are tumor-promoting inflammation and genome instability and mutation. They call these “enabling characteristics”, and a lot of people think that the completion of all these other steps would be very, very rare without these two characteristics, particularly the genome instability

and mutation.

The networks of the hallmarks will differ from cell to cell, tissue to tissue. You won't have the same hallmarks in every tissue. Every tissue won't have all of these. The timing will differ too. It just depends on the cell type, the tissue, and even the micro environment will definitely play a role.

So metals as carcinogens, the problems and importance. You've seen several of the other speakers go over this, so I won't go into very much detail, but just to emphasize the importance of these in metals toxicology. Three-fourths of all elements are metals or metalloids. They're ubiquitous in the environment. There's wide use and distribution. Anthropogenic sources, a large number of industrial processes, natural activities such as the arsenic in the drinking water. Persistence in the environment - can be neither created nor destroyed, forms change, kind of moving around throughout the biosphere. They're only sparingly recycled once used. More on their persistence is they're nonbiodegradable, very long half-lives for many of them. You can see with cadmium

it can be several decades; lead, several months. And these long half-lives can lead to the accumulation over an entire lifetime which really makes their potential as a human carcinogen of particular concern. And there are also several known, probable, or suspected human metallic carcinogens which we'll get to in a second.

The uniqueness of metals as carcinogens - very highly diverse group of agents, generally do not require metabolic activation. Often times the ionic form is already active and it will serve as the carcinogen. They're often highly tissue-specific, high concentrations at the target site. Susceptibility factors may be different from tissue-to-tissue and so on.

We've talked about essential metals. Some of these are also carcinogenic. Chromium, I would agree with Dr. Costa, this may not be an essential metal, but there are some who believe it is, so we'll put that in quotes. Chromium 3 is essential. Chromium 6 is highly carcinogenic. Similar with nickel. Some say it's essential. Can also form respiratory tract tumors; and iron is a possible liver carcinogen and is associated

with colorectal cancer.

On the other hand, some carcinogenic metals are also very effective chemotherapeutics. Dr. Costa mentioned arsenic trioxide. There's also platinum in the form of cisplatin. And there are studies that show cadmium can also have anticancer effects. We'll get into those three examples in a little bit, with a little bit more detail.

So what are the metals that are carcinogenic to humans? There are different regulatory agencies that sort of determine this for us. In the United States, there's the NTP, the National Toxicology Program, which puts out their report on carcinogens. The EPA, FDA and NIOSH. And then there's the International Agency for Research on Cancer, or IARC, and they put out their monographs.

For this talk, we're going to focus mainly on IARC's definitions, as well as that of NTP. Definitions and their criteria are very, very similar; and you'll see that most of the known human carcinogens are basically the same between the two agencies, as well as the possible or probable carcinogens,

reasonably anticipated to be carcinogens.

So IARC's definitions, they put them into different groups. Group one is the cariogenic to humans, known human carcinogens, and you see all of those listed here. Arsenic, beryllium, cadmium, chromium 6, nickel, and their respective compounds. You also have group 2A, which are probably carcinogenic - lead, cisplatin, indium phosphide. And group 2B is possibly carcinogenic -- this is cobalt and iron.

The RoC definitions. You've got this in front of you. I won't read through all of the criteria, but you see the known human carcinogens -- It's essentially same as IARC.

The reasonably anticipated to be human carcinogens, you see the criteria here and then the bottom line you can see lead, cisplatin, indium phosphide and cobalt. Basically the same ones as IARC considered possible or probable human carcinogens.

The chronology next. The very first report of a metal as carcinogen was made in 1880 by a physician named Hutchinson. He noticed skin tumor formation in people who were going through arsenic therapy for skin

disorders. So that was about 130 years ago. 60, 70 years later we had our first epidemiology studies. And then about 50 or 60 years further in the '90s and 2000s, we had our first good animal studies. It's kind of unusual to have such a long time between good human data and rodent data, and this may be due to the time of exposure of the rodents to arsenic.

A little earlier -- in the '30s we had chromium and nickel. These were the first two recognized by IARC as human carcinogens. And about 30, 40 years later we had all the good evidence. Beryllium, first reports in 1946. Cadmium and lead, it's a little bit later in the '60s. And then the good evidence in humans and rodents in the early of '80s.

So the known human carcinogens, and these are just listed in alphabetical order.

Arsenic and its compounds, it's a known human carcinogen by both the regulatory agencies, IARC and NTP. It's number one on the Agency for Toxic Substances and Disease Registry Substance Priority List. That's probably why you see so much work being done on arsenic. Over 100 million people exposed

worldwide. Lots of target sites in humans. The definite sites are skin, lung, and urinary bladder; possible sites are liver, kidney, and prostate. Several routes of exposure, both environmentally, occupationally, a lot of studies over the past several years with transplacental exposure. Multiple carcinogenic species. Multiple mechanisms. As I mentioned, it's a very effective chemical therapeutic, mainly arsenic trioxide with acute promyelocytic leukemia. It appears that at least one of the mechanisms, perhaps the main mechanism, is that the arsenic trioxide can target and basically eradicate the leukemic stem cells in the acute promyelocytic leukemia, and that essentially cures the disease.

Beryllium and its compounds, again a known human carcinogen by both agencies; number 43 on the priority list. One exposure problem with beryllium is a long residence time, greater at least 20 years, at the target, in this case lung; environmental and occupational routes of exposure, target sites definitely in the human lung; osteosarcomas in rabbits; and again, several mechanisms. You'll notice a lot of

these mechanisms between all of these metals are shared. There's a lot of common mechanisms between all of these metals.

Cadmium and its compounds are considered known human carcinogen by both agencies, number seven on the list. A big problem with cadmium as I mentioned earlier, very long half-life, making carcinogenic potential of particular concern because of accumulation throughout the years. Several routes of exposure, many are the same as with all the other metals. Target sites - definitely in the lung in humans, a lot of possible sites in humans as well; and again, several different mechanisms.

Chromium 6 compounds. It's one of the first two classified by IARC, as I mentioned the other being nickel. Routes of exposure are very similar to other metals. Target sites are the lung. And as Dr. Costa mentioned, there's a lot of new data coming out that possibly all tissues could be targeted. Many mechanisms involved. I don't have epigenetic mechanisms here, but as Dr. Costa mentioned, it definitely has epigenetic mechanisms.

Nickel and its compounds. This one is a little different. It's the nickel compounds that are known human carcinogens, as defined by IARC and RoC. Metallic nickel is only a possible by IARC, reasonably anticipated by RoC. Routes of exposures, again very similar with other metals; occupational, environmental and in this case transplacental. This is one case where the ionic form is the likely cariogenic species; a few different target sites and many different mechanisms.

Inorganic lead, this is a probable human carcinogen. And, again, very similar routes of exposure with other metals, both occupationally and environmentally. Another one similar to nickel, it's the ionic species that is the probable active carcinogen. Several target sites. And, again, several mechanisms.

Cisplatin, another probable carcinogen. Routes of exposure, a little different in this case, the medicinal and occupational with medical treatments, the drug administrations, is how people are exposed to cisplatin. A lot of target sites in rodents. In

humans there's only inadequate evidence based on concurrent therapies. And this is another one that's a highly effective chemotherapeutic, sometimes called the penicillin of cancer treatment. And I forgot to mention that cadmium is another that's been shown to have anticancer effects in rodents. From my poster on Tuesday afternoon in the stem cell session, you can see some mechanisms that we are coming up with as to why cadmium may have these anticancer effects. It appears to be very similar to the arsenic trioxide with the targeting of the stem cells and the cancer stem cells. So if you're interested, Tuesday afternoon, I'll be glad to talk to you about my poster.

Indium phosphide, another probable human carcinogen. Again, routes of exposure are very similar to the other metals. Occupational, environmental exposures, target sites mainly in rodents in this case.

And then the possible carcinogens, cobalt and iron. You see the rankings or the groups, the definitions by each of the agencies here and why they've defined them in that way.

There are several factors that modify metals

carcinogenesis. Metal-to-metal interactions is a very important one. Some of this has been discussed in the previous talks. But just to reiterate, just to show you the importance of these effects, several metals, zinc, calcium, magnesium can inhibit, prevent or even antagonize metal-induced tumorigenicity, just by reducing metal uptake, inhibiting the effects on DNA, attenuating the toxic effects of the metal.

Metallothionein synthesis appears to be a zinc-related mechanism. Metallothionein will bind the toxic metal, cadmium, for instance, and attenuate those toxic effects. And iron tends to enhance nickel-induced carcinogenesis.

An example of essential metal deficiency in enhancing tumorigenesis, is dietary zinc deficiency and cadmium-induced tumors in rat testes. You'll see in the middle blue-shaded row, these are zinc-deficient rats and rats with adequate zinc levels not treated with cadmium, and essentially the same levels of adenoma or hyperplasia formation. However, you see a much higher incidence of adenomas in the zinc-deficient rats treated with cadmium. And while this is an

adenoma, not a malignant tumor, a lot of times these will progress to a malignant adenocarcinoma, particularly if cadmium treatment was continued, which would greatly increase that chance.

Other mechanisms, many of them are metal, tissue or cell-type specific. As I mentioned, you see many common mechanisms are shared between the different metals. Most of them involve DNA damage repair, a lot of epigenetic effects. There are a lot of adaptive mechanisms. This involves changes in the uptake or the efflux or excretion of metals from the cells. A lot of times the cells will acquire a tolerance to the metal. They'll become apoptosis-resistant to that metal. Also enhanced metal efflux will help remove the metal from the cells. Metal sequestration, sequestered lead and metallothionein with cadmium. Oxidated stress response involving several factors, NRF2, hemoxygenase, superoxide dismutases and so on. But many times this acquired adaptation, it sounds good, but it may actually facilitate cancer formation. As an example here on the right involves several of these mechanisms. You see the parental cell line, the human prostate cell

line mature heterogenous population in the black bars, and the isogenic stem cells that were isolated from this cell line, both treated with sodium arsenite. You can see a much higher LC50 in these stem cells.

So the stem cells -- very common with toxicants -- are much more resistant compared to their mature, more differentiated counterparts. So this is a kind of cell-type mechanism.

After only about six weeks of chronic exposure, both cell lines you can see did adapt. LC50's increased, about doubled in the parental cell line. However, that didn't increase anywhere near as much as it did in the stem cell lines. So both cell lines adapt, but the stem cells appear to hyper-adapt to arsenic. And a few more weeks of chronic exposure, both of these cell lines do become malignantly transformed. These stem cells are transformed in approximately half the time it takes to transform the parental cell line. So even though they're hyper-adaptable, they may become transformed much, much quicker. So this acquired adaptation is not always beneficial.

Mimicry, you've heard a couple of the other speakers talk about this. Again, I'll reiterate just to emphasize its importance. It's a very key mechanism in the toxicity of metals. It allows the metals to gain access into the cells and disrupt critical cell functions, metabolism, cell signaling functions. It's usually at the transport level, sort of fools the cell into thinking it's the essential metal, gets inside the cell and it exerts its toxic effects.

There are several examples. Cadmium, copper, and nickel mimic or replace zinc; thallium mimics potassium; magnesium mimics iron; and arsenate and vandate mimic phosphate. Lifestyle factors are other things that modify metal carcinogenesis. This really isn't a mechanism per se, but I wanted to include it. Factors like smoking, alcohol consumption, and diet nutrition play a major role in metals carcinogenesis in humans.

Strain and species differences is another factor that modifies metal carcinogenesis. Here is cadmium carcinogenesis in rodents. Injection site sarcoma on the top row in rats, two different species,

F34 a little over 60 percent tumor incidence, whereas the other strain only about a ten percent incidence. Lymphomas in two different mice strains, NFS at almost a 70 percent incidence, whereas the BALB/c mice, only 21 percent incidence. And you'll notice in the legend, the bottom line here, the control lymphomas in the BALB/c mice was also 21 percent. So that sort of wipes out the 21 percent incidence in the cadmium-treated mice. That makes the difference between the strains that much greater.

Genetic variability is another factor that modifies metals carcinogenesis. You've seen several examples already with arsenic and the metabolism of arsenic. Major players in this are polymorphisms in the AS3MT and also in the GST omega 1. Differences in DNA repair genes play a big role in arsenic-induced skin cancer, bladder cancer. Cadmium and metallothionein levels, and there's also effects on non-cancer diseases. This is not just all cancer. I'm focusing on cancer, but a lot of other non-cancer diseases where genetic variability plays a big role. You see several examples listed here.

Metallothionein is a very important player in metals toxicity. Human expression is highly variable. In some tissues between different populations, there can be up to or even greater than 100 fold differences in blood levels. Very key factor in metal homeostasis and detoxification, high affinity for metals. Deficiency can predispose to metal toxicity or cancer by several metals, and there are a lot of studies on MT-null mice, showing the null mice are much more susceptible to cancer induced by several different metals compared to the wild type mice.

Another factor is the route of exposure. The portal of entry is often the organ most affected. As you saw in a lot of the metal slides, talking about routes of exposure, the big one is inhalation. This is the most important the occupational route. In most cases with inhalation you'll get metal particles in the lung space and wind-up developing lung cancer. Tobacco smoke obviously is a big one that contains arsenic, cadmium, nickel and lead.

Ingestion of foods and liquids, you heard several times about the arsenic in the drinking water.

Dr. Costa mentioned the arsenic in the rice, just two very good examples for routes of exposure with ingestion. And dermal exposure, it's a little more unusual than the other ones, but it does occur.

Life stage during exposure. Young subjects are often more sensitive. There are a lot of studies coming out the past decade or so of transplacental carcinogenesis. A lot of times transplacentally-exposed rodents show much, much higher incidences of cancer formation later in life, long after the exposure has ended. Early life exposure: there are some very good studies by Alan Smith's group on a population in Antofagasta, Chile. Also with lead compounds, greater risk in young versus adults. And whole life exposure it appears -- and I'll go over examples of all of these in a bit -- but it appears that transplacental exposure dictates -- or may dictate the target site whereas exposure during other periods will promote or even enhance the carcinogenesis.

So as I mentioned earlier, you can't really, in my opinion, have a talk about cancer without talking about stem cells, without talking about cancer stem

cells, and this will bring us to the cancer stem cell hypothesis. So just to define each of these, very simple definitions in this case, a stem cell is a cell that can regenerate all cell types of any tissue if it's an embryonic stem cell or all cell types of a given tissue if it's a progenitor cell or adult stem cell. Cancer stem cells share pretty much all the characteristics with a normal stem cell. Only a cancer stem cell obviously can transfer or form tumors upon transplantation into a recipient animal. And the cancer stem cell hypothesis basically states that cancer stem cells are derived from the malignant transformation of normal stem cells or their close, partially differentiated progenitor cells, to become cancer stem cells, that then drive the carcinogenic process.

So stem cells and metals carcinogenesis, I will focus on arsenic and cadmium, mainly on arsenic. Most of the studies have been done in arsenic so far, but there's more coming out pretty much every week.

Stem cells appear to be much more resistant to arsenic. As you saw in the slide earlier, they're also

hyper-adaptable. This is very common with many other toxicants, not just arsenic. Stem cells also seem to be much, much more resistant compared to their mature differentiated counterparts. It appears to disrupt the normal differentiation, the arsenic exposure. You see in the top here, these are human stem cells exposed to arsenic for 9 weeks up to 18 weeks. And looking at P63 protein levels, P63 is a very good -- these are human prostate stem cells. P63 is a very good marker of human prostate stem cells.

So you can see at 9 weeks, almost a depletion of the P63, but by 18 weeks when these cells were malignantly transformed, this P63 expression is back up to control levels. So there's some sort of aberrant differentiation being caused by arsenic.

The arsenic also appears to lead to the formation or overproduction of cancer stem cells. This is in comparison with other carcinogens. That's the example here on the bottom. You have the RWPE-1 cell line. This is the non-tumorigenic human prostate epithelial cell line. So we're using this as our control. It's been malignantly transformed by MNU,

cadmium and arsenic. So these are all isogenic cell lines. And you can see only the arsenic caused an increase in cancer stem cell formation as measured by the formation of the spheres which we call prostaspheres. Very common characteristic of stem cell lines and cancer cell lines, they form these spheres which are just free-floating clusters of viable cells that are generally highly enriched in stem cells or cancer stem cells. So the larger number of spheres, obviously the larger number of, in this case, cancer stem cells. And we've seen similar results in prostate, skin and kidney with arsenic exposure on stem cells. Cadmium in stem cells appears to be a little different. Seems to inhibit proliferation and self-renewal. Cells can acquire stem-like characteristics during chronic exposure, which was shown in pancreas cells. A lot of stem cell markers show rather large increases after chronic exposure and when these cells appear to be transformed. However, the sensitivity to cadmium may depend on the stage of differentiation; and again, going back, I mentioned my poster, if you go to my poster, you'll see good

examples of this and some possible mechanisms involved. It appears that the more undifferentiated the cells are, the more sensitive they are to cadmium.

So now jumping into the developmental origins of adult disorders. This all started with this guy up here, that's David Barker. In about 1989, he noticed a very strong association between low birth weight and a greater risk for coronary heart disease later in life. He's published over 400 papers on this topic. He's seen similar results from all of these disorders listed on the bottom. So I think it was in 1995 the British Medical Journal named his hypothesis, what he's called fetal programming, they renamed it to the Barker hypothesis, and it's also referred to, a lot of times, as the fetal origins of disease or the fetal origins of adult disease. And even Time Magazine two or three years ago called this “the new science.” And you don't have this Time Magazine cover in your guide for copyright reasons, I guess.

So, some good examples of developmental origins of adult disorders, again focusing on cancer in this case. These are in humans. Early life exposure

of a population in Antofagasta, Chile, I mentioned with Alan Smith's group, they found that these early life exposures greatly increase cancer mortality rates in those born around the time of the peak exposure. This was in liver, lung and renal cancers. And they just published a very good review article on all of these epidemiological studies they have done. Came out maybe a month or two ago. So if you're interested in these studies, I would suggest reading that review article.

Another good example is infants in Okayama, Japan, they were exposed to -- in 1955, exposed to arsenic contaminated milk powder. And those that were exposed to this contaminated milk powder, later in life showed greatly increased rates of leukemia, as well as skin, liver and pancreatic cancers, and greatly increased mortality rates from the Leukemia and the pancreatic cancers.

Some examples in rodents. This is a transplacental arsenic exposure I was talking about earlier. The transplacental exposure from gestation day 8 to 18. These lead to cancers much, much later in life. It can also predispose to other carcinogens and

lead to a cancer stem cell overabundance in the arsenic-induced tumors in several of these. I think I got the wrong slide up there again. The cancer is much later in life, in the bladder, skin, lung, liver, and kidney. It's important to point those out, because those are also the target sites in humans exposed to arsenic. Some very good tissue-tumor concordance between these transplacental rodent studies and human exposure.

As far as predisposing to other carcinogens, you'll see the example in the immunohisto chemistry on the top. The label is not here, but it's in your guide. These -- this is on the left, this is the arsenic plus TPA, exposed mice. You see a much, much higher number of CD34 positive skin cancer stem cells. CD34 is a very good marker for skin stem cells and cancer stem cells. And this is in comparison to the tumors formed in the TPA alone mice.

Early life exposure. I mentioned that with the lead compound is a greater risk in the young versus adults. And whole life exposure, the transplacental may dictate target site while other periods promote.

This example is here on the bottom. The fetal only or the transplacental exposures in the black bars, whole life exposure in the gray. You can see all the same tissues, but the whole life compared to the transplacental only, you see much, much higher incidence levels, and in most cases, the tumors were much more aggressive in the whole life exposed mice. And the cancer stem cell overabundance that you see in the images to the right, the two control tumors in liver and lung, top and bottom, spontaneous tumors, these are stained with aldehyde dehydrogenase 1A. It's a very common cancer stem cell marker for both of these tissues. And compared to the control spontaneous tumors, you see much, much higher levels in the arsenic-induced tumors.

And again, it's not just cancer. There's a lot of data out, coming out, on developmental origins of other disorders. Arsenic and bronchiectasis, early onset atherosclerosis, deficient lung function; cadmium, lower childhood intelligence, mental retardation, decreased birth weight; lead in the HPA axis dysfunction which is associated with obesity,

diabetes, hypertension. Smoking during pregnancy, this is another big one that leads to impaired fertility, obesity, hypertension, neural behavioral deficits. It's a little trickier with smoking just because there's a lot of different metals in cigarette smoke, so, you don't really know if it's the effect of one of these metals causing all of these or a combination of the metals causing all of them. It's a little trickier with the smoking.

So what is the role of stem cells in the developmental origins? Well, stem cells are most active and abundant during development. All of these processes, organogenesis, proliferative growth, cell differentiation, they all are intimately involved with stem cells. Stem cells play a key role in all of these. And a very good example of more stem cells at exposure leading to more later-life cancers is the A-Bomb survivors and breast cancer. Women exposed to the A-Bomb radiation had a much higher incidence level of breast cancer compared to those not exposed. And the woman exposed during late adolescence, the time when breast stem cells are most abundant, showed by far

the highest incidence of breast cancer. So it's a good example of more stem cells at exposure, leading to more later-life cancers.

And it's the long-lived nature of the stem cells, their ability to remain quiescent until activated or promoted, their ability to maintain or retain the capacity for self-renewal and the capacity for differentiation. It's thought that this is the reason early life is often sensitive to chemical carcinogenesis. It's hitting the stem cells early on. And then later on in life they lead to cancer formation.

Do stem cells play similar roles in the other disorders? I believe it's reasonable to suspect that they do. I really can't think of any other cell type that's going to be around in the fetal stage all the way up to 20, 30, 40 years later during disease manifestation. But this sort of is just my opinion, I think it definitely requires further research.

So to conclude very quickly, almost out of time, metals are very complex. Carcinogenesis is very complex. So you put these together, metals and

carcinogenesis is very, very complex. Many challenges. Metals are very highly unique. Many of them are carcinogens. You saw the different mechanisms involved, not just in my talk, but all the talks today, and there's definitely many factors that can modify the carcinogenicity of metals. Developmental origins of adult disorders, this is definitely, in my opinion again I guess, a very important emerging field that warrants further research. Early life exposures definitely play a role in metals carcinogenesis. We've seen examples in humans and rodents in this talk. And stem cells appear to be involved, at least with some of the metals in cancer. The involvement, again, with stem cells with the other disorders definitely needs more research. And I believe that's it. Yep. Thank you.

DR. HUGHES: So are there any questions for Dr. Tokar?

FROM THE AUDIENCE: Hi. You had mentioned the importance of the micro environment. I think Minam Bassel’s (ph) work has shown that DNA mutation suggested at least that that might not be sufficient

for cancer development, that the cellular shape and adhesion is important as well. And I think that divalent cations are essential for endocrine function. So I'm interested to know if any work has been done to look at the impacts of metals and cell adhesion and cell shape that might progress for cancer development in addition to the DNA mechanisms?

DR. TOKAR: I don't know off the top of my head. I'm sure there are studies. I can't think of any off the top of my head, but I'm sure there are studies looking at that. Sorry, I don't have any examples.

FROM THE AUDIENCE: Fair enough. Thanks.

DR. TOKAR: Yeah, maybe another cheap plug from our group, from Mike Waalkes' group, is one of the SOT post-doc papers of the year by Yuanyuan Xu, she showed a very good example of -- of microenvironment effects on the transformation of cells. She used co-culture method to show how malignantly transformed cells without any contact -- non-contiguously, I guess, can transform normal stem cells into a cancer phenotype. And one of the factors showed a lot of

epithelial-mesenchymal transition factors. Vimentin went way up, E-cadherin goes down.

FROM THE AUDIENCE: It's my understanding that the transcription factor NTF zinc transcription factor binds zinc and sort of drives transcription of metallothionein, which as you mentioned, can sort of sequester toxic metals like cadmium.

Cadmium actually has a higher affinity for that transcription factor and can also drive that same transcription activation. And so, I was just wondering what the mechanism was or, you may or may not know the answer to this, or suggested mechanism of the decreased zinc levels predisposing to carcinogenesis with cadmium. Because as I said, you would think if, you know, cadmium also drove the reaction and upregulated metallothionein that would help, you know, reduce the levels of cadmium. And if zinc wasn't there actually there would be even more for it to bind to the cadmium.

DR. TOKAR: Yeah, I don't know the exact mechanism. We had some evidence of mechanisms on my poster where the zinc transporters are -- during cadmium exposure, they're going way up. And we think

that ones that efflux the zinc, it appears that the zinc is being effluxed out of the cells. Cadmium is coming in a lot more. MT levels stay about the same. So, getting rid of the zinc, taking in more cadmium, that might be at least part of the mechanism, but I don't know the exact.

FROM THE AUDIENCE: All right. Thanks.

DR. HUGHES: It's noon. So the speakers will be up here for a few minutes if you have any other questions for them, and I thank you for attending. And I'd like to thank my fellow speakers for preparing the talks today. I learned a lot. I hope you did, too. And thank you again.

(Whereupon, the presentation was concluded.)