Molecular Mechanisms of Nickel Carcinogenesis

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Carcinogenic, water-insoluble Ni compounds are phagocytized by cells; and the particles undergo dissolution inside the cell, releasing Ni ions that interact with chromatin. Ni produces highly selective damage to heterochromatin. The longest contiguous region of heterochromatin in the Chinese hamster genome is found on the q arm of the X chromosome, and this region is selectively damaged by Ni. More than half of the male mice in which there were Ni-induced transformations of Chinese hamster cells exhibited complete deletion of the long arm of the X chromosome. The introduction of a normal X chromosome into these cells resulted in cellular senescence, suggesting that the Ni interacted with Chinese hamster genome to inactivate a senescence gene. Investigations were conducted into the mechanisms by which Ni produced damage to chromatin. Ni ions have a much higher affinity for proteins and amino acids than for DNA (by five to seven orders of magnitude). Therefore, Ni interacted with chromatin because of the protein present, not because of its reactivity for DNA. Studies have shown that Ni produced an increase in oxidative products in cells as indicated by oxidation of the fluorescent dye dichlorofluorescein; Ni has also been shown to produce oxidation of proteins in cells, as measured by carbonyl formation. Ni cross-linked certain amino acids and proteins to DNA. These covalent cross-links were not dissociated by EDTA and are inconsistent with direct Ni involvement, but they are consistent with Ni acting catalytically. Using subtractive hybridization, we have isolated a number of clones that are expressed in normal but not in Ni-transformed cells. One of these genes is thrombospondin. The levels of thrombospondin as measured by mRNA and monoclonal antibodies were very low in Ni-transformed cells compared with normal cells. The gene appeared to be intact, based upon restriction enzyme analysis with Southern blots. We have demonstrated, using the promoter region of thrombospondin, that transcription of a CAT reporter gene is lower in nickel transformation compared to normal cells. We are currently investigating mechanisms for this down regulation and testing the possibility that this may involve the loss of a senescence gene which affected the transcription of genes such as thrombospondin. — Environ Health Perspect 102(Suppl 3):127–130 (1994).

Key words: thrombospondin, oxygen radicals, gene expression, heterochromatin

Introduction

In recent years the molecular mechanisms of Ni carcinogenesis have enjoyed some clarification. The understanding of why certain particulate Ni compounds are such potent carcinogens in experimental animals, whereas water-soluble Ni salts are not carcinogenic may be explained by the fact that water-soluble Ni salts do not enter cells, whereas certain carcinogenic particulate Ni compounds are actively phagocytized by target cells of transformation, allowing Ni ions to reach high levels in the cell and producing genetic damage associated with carcinogenesis (*1*).

A second major revelation comes from a greater understanding of the intracellular mechanisms by which Ni induces its carcinogenesis. Ni ions have been shown to form oxygen radicals and to increase oxidants in cells catalyzing the formation of covalent cross-links of proteins and amino acids to DNA (*2,3*). Such cross-links are considered important in Ni carcinogenesis because they represent a bulky lesion that is not readily repaired (*1*).

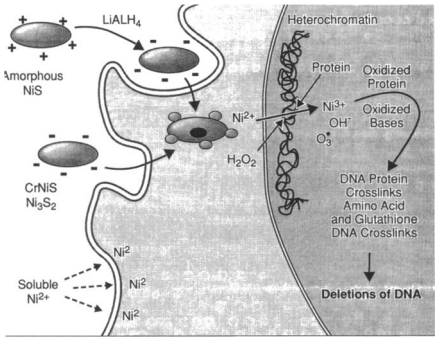

Figure 1. Summary of the events involved in Ni carcinogenesis, demonstrating the phagocytosis of Ni sulfide particles, their dissolution inside the cell, and their interactions with chromatin. Soluble Ni salts do not enter cells very well; therefore, they are not potently carcinogenic.

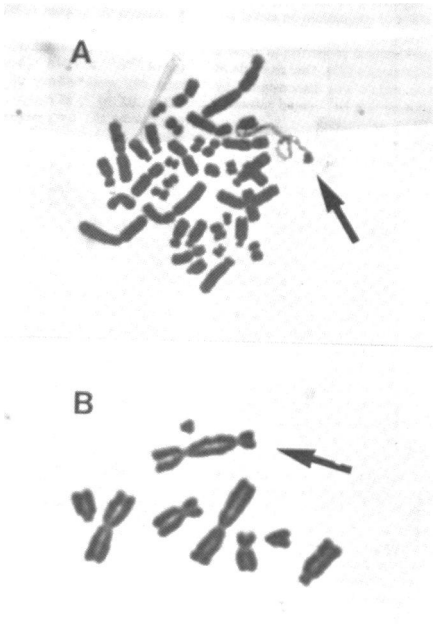

Figure 2. Selective damage that nickel produced on the long arm of the X chromosome. This region is entirely heterochromatic and represents the longest contiguous region of heterochromatin in the Chinese hamster genome. Ni binds very tightly with protein, compared to DNA; this fact may explain the selectivity of Ni interaction with protein-rich heterochromatin.

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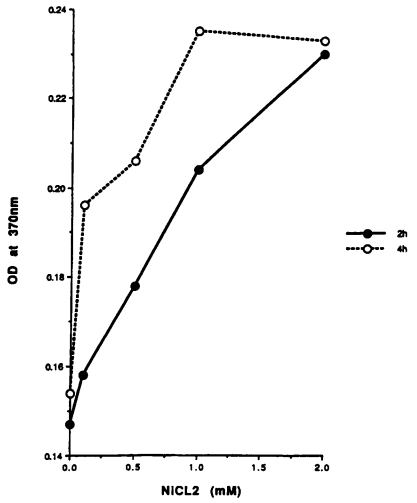

Figure 3. $NiCl_2$-induced protein oxidation in cultured Chinese hamster ovary (CHO) cells CHO cells were treated with indicated concentrations of $NiCl_2$ for 2 or 4 hr in SGM. The carbonyl content of oxidized protein was determined (*11*). The values are means of three separate determinations.

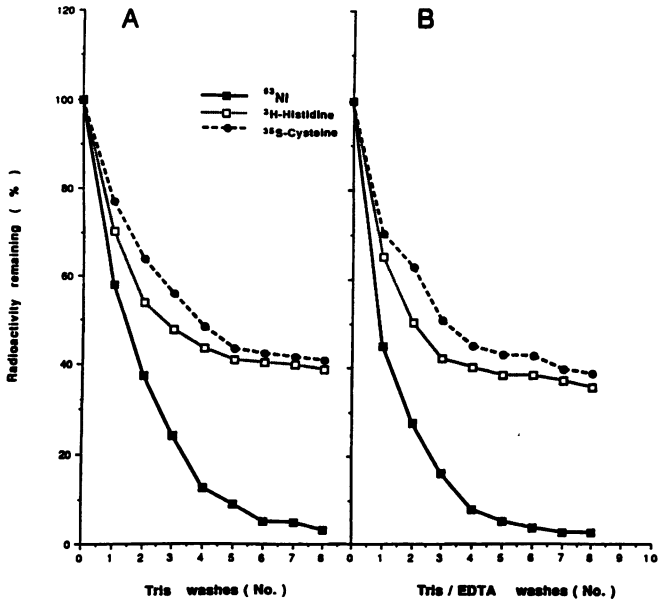

Figure 5. Stability of $NiCl_2$-induced amino acid DNA cross-link and ^{63}Ni DNA binding in CHO cells. CHO cells were labeled with [^{35}S]cysteine and [^{3}H]histidine 24 hr prior to receiving exposure to 1 mM $NiCl_2$ for 20 hr (for amino acid DNA crosslink) or to 1 mM $^{63}NiCl_2$ (20 mCi/ml) for 20 hr (for ^{63}Ni DNA binding). Each DNA sample was incubated with 10 mM Tris, pH 8.0 (A) or 10 mM Tris and 20 mM EDTA, pH 8.0 (B) for 30 min at room temperature, as indicated in the figure. After each incubation, the sample was filtered through Ultrafree-MC filter units (Millipore) and the disassociated amino acid residues and ^{63}Ni radioactivity were determined.

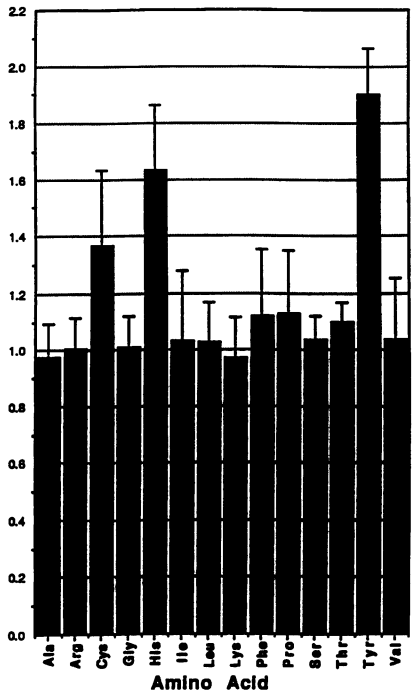

Figure 4. Comparison of the residual amino acids associated with DNA following $NiCl_2$ treatment of intact CHO cells. CHO cells were labeled with any one of the indicated radioactive amino acids for 24 hr prior to receiving a 5 hr exposure to 0.5 mM of $NiCl_2$ in SGM. DNA was isolated by proteinase K/phenol/chloroform extraction. Residual cysteine associated with DNA was determined as previously described (*12*). Each value is the mean ± SEM for three determinations.

The fact that Ni might catalyze these lesions in a "caged" manner, i.e., when it is bound to chromatin proteins near the DNA, further suggests the importance of this mechanism. The nonrandom damage produced by Ni in heterochromatin suggests that Ni may generate radicals in certain locations within the genome and produce its damage selectively (*1,4*). Because it does not catalyze an abundance of radicals, as would copper or iron, there is less cytotoxicity, and genetic damage is localized (*2*). The present study summarizes major advancements in the last few years in our laboratory on the molecular mechanisms in Ni carcinogenesis.

Phagocytosis of Nickel Compounds and Interaction with Heterochromatin

Figure 1 summarizes the events involved in Ni carcinogenesis and demonstrates the phagocytosis of Ni sulfide particles, their dissolution inside the cell, and their interactions with chromatin. Soluble Ni salts do not enter cells very well, therefore they are not potently carcinogenic (*1*).

The ability of certain particulate Ni compounds to enter cells explains the carcinogenic potency of these compounds. Following phagocytosis, the particles dissolve, Ni^{+2} ions are released, and selective damage induced is in heterochromatic regions.

Figure 2 shows selective damage that nickel produced on the long arm of the X chromosome. This region is entirely heterochromatic and represents the longest contiguous region of heterochromatin in the Chinese hamster genome (*4*). Ni binds very tightly with protein, compared to DNA; this fact may explain the selectivity of nickel interaction with protein-rich heterochromatin (*5*).

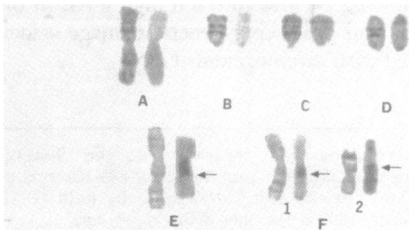

Figure 6. G- and C-banding profiles of the altered X chromosomes from the Ni-transformed cells. Male Ni-transformed cells exhibit a complete deletion of the long arm of the X chromosome. Introduction of a normal X chromosome into Ni-transformed cells resulted in senescence of these immortalized tumorigenic cell lines, suggesting that one of the genes inactivated during nickel transformation was a senescence gene. From Conway and Costa (*4*); with permission.

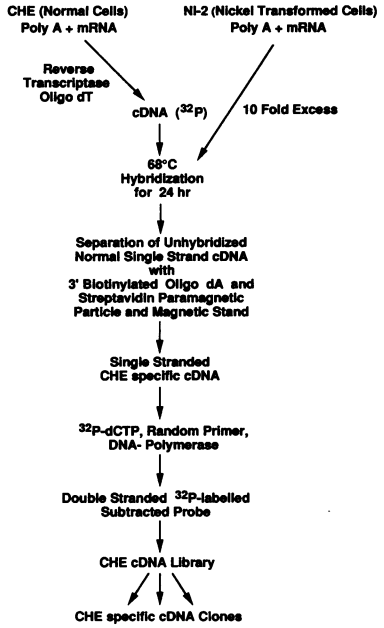

Figure 7. Scheme for obtaining CHE-specific cDNA subtractive clones.

Mechanisms by which Nickel Damages DNA

Ni compounds previously have been shown to increase oxidants in cells since they oxidize the dye dichlorofluorescein diacetate to a fluorescent product whose formation is catalyzed only by very strong oxidants such as hydrogen peroxide (*6*). We also demonstrated that Ni produced increased protein oxidation as determined by carbonyl formation. Figure 3 shows increases in the formation of protein carbonyl induced by Ni

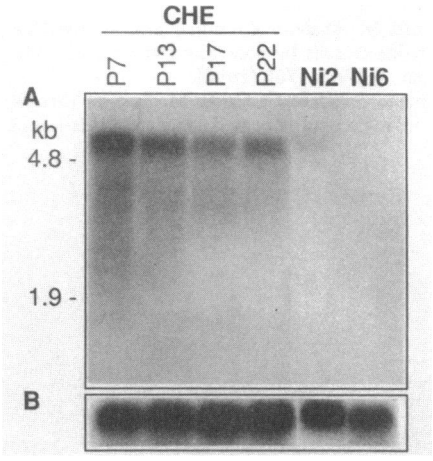

Figure 8. Northern blot analysis of the mRNA expressed in normal CHE cells at different passages compared with the expression of thrombospondin mRNA in Ni-2 and Ni-6 transformed cells (Section A). Hybridization with β-actin confirms equivalent loading of mRNA in all lines (Section B6). From Salnikow et al. (*13*); reprinted with permission.

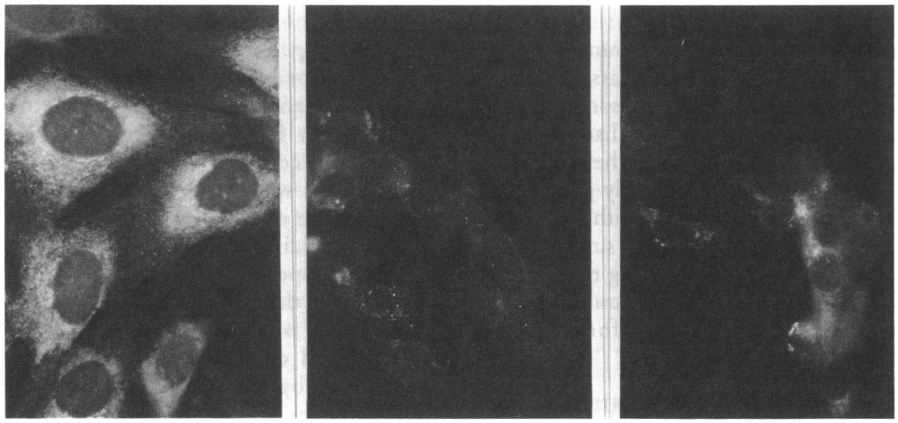

Figure 9. Monoclonal antibody staining of thrombospondin in normal cells (A) or Ni-2 (B) or Ni-6 (C) transformed cells. Cells were stained with a primary monoclonal antibody against human thrombospondin, then were incubated in an FITC-tagged second antibody. From Salnikow et al. (*13*); reprinted with permission.

compounds in intact cells. Ni also increased oxidation of cellular proteins in vitro in the presence of hydrogen peroxide. This increase in oxidants formed in cells could be responsible for a variety of Ni's intracellular effects. One effect attributed to increased oxidants is the covalent cross-linking of certain amino acids, such as histidine, cysteine, and tyrosine and to DNA, as shown in Figure 4. These cross-links are covalent since they resist extraction with sodium dodecyl sulfate (SDS). Their covalent character and resistance to EDTA extraction negates the direct participation of of Ni^{+2}.

Figure 5 shows that the cysteine and histidine cross-linked with DNA were more stable than the Ni associated with DNA, suggesting that the Ni caused the cross-linking of these amino acids by a catalytic mechanism rather than participating directly in the cross-links. The ability of nickel to induce formation of oxidants, oxidize protein and DNA, and induce cross-linking of amino acid and proteins to DNA probably contributed to the striking damage observed in heterochromatin in Figure 2 and subsequent deletion of this heterochromatic region, as discussed in the next section.

Deletion of a Senescence Gene during Nickel-induced Transformation of Cells

Ni-induced cellular transformation of male Chinese hamster embryo cells is associated with a complete deletion of the long arm of the X chromosome, the initial site at which Ni produced damage (Figure 2). Figure 6 shows male Ni-transformed cells that exhibit a complete deletion of the long arm of the X chromosome. Introduction of a normal X chromosome into Ni transformed cells resulted in senescence of these immortalized tumorigenic cell lines, suggesting that a senescence gene was inactivated during Ni-induced cellular transformation. (*7*). However, introduction of a normal X chromosome into Ni-transformed cells without this complete chromosome deletion also induced senescence. Since the region deleted was entirely heterochromatic, these results suggest that the senescence gene is probably not located in the region of obvious deletion. The inactivated senescence gene in Ni-transformed cells was under methylation control, as

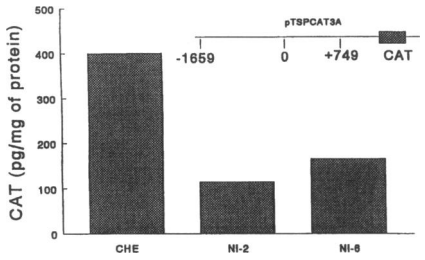

Figure 10. The activity of the thrombospondin promoter linked to a CAT reporter gene in normal and Ni-2 or Ni-6 transformed cells. The pTSPCAT3A construct shown in Figure 6 contained region –1659 to + 749 of the thrombospondin promoter and the first exon of the thrombospondin gene. The pTSPCAT7A construct contained a smaller portion of the thrombospondin promoter region and first exon of the gene (–234 to +749). For examination of CAT reporter gene activity in normal and Ni-transformed cells, plasmid DNA was introduced into cells, CAT and β-GAL activity were measured by Elisa kits following transient transfection of the vectors into the cells. Values are the means of duplicate determination from one of three representative experiments.

demonstrated by experiments in which the gene was transferred to Ni-transformed cells by microcell fusion from mouse cell hybrids at early and late passage (*6*). At late passage, the X chromosome harbored in the mouse A9 cell lines had less senescence activity than when the X chromosome was transferred at early passage (*6*). Late-passage cells treated with azacytadine exhibited senescing activity similar that of early passage cells, suggesting that the gene was under methylation control (*6*). A senescing gene was also found on the human X chromosome (*8*).

Genes Inactivated during Nickel-induced Transformation

A newly developed scheme for subtracting mRNA from normal cells with Ni-transformed cells is illustrated in Figure 7. A number of clones present in normal cells but deleted in Ni-transformed cells were obtained. One of these clones was highly homologous to the thrombospondin gene. Figure 8 shows the mRNA expression of the thrombospondin gene in normal cells and the much lower levels of mRNA in Ni-transformed cells (*13*). These Ni- transformed cells were able to form tumors in nude mice and grew in soft agar (*4*). Studies utilizing monoclonal antibody to thrombospondin (Figure 9) demonstrated that the protein was present in normal cells but greatly reduced in Ni-transformed cells. To investigate the mechanism by which thrombospondin may be turned off in Ni-transformed cells, the promoter to thrombospondin was linked to a CAT reporter gene and transient transfections were conducted.

As shown in Figure 10, the transcriptional activity of thrombospondin in normal cells was much higher than that of Ni-transformed cells, suggesting down regulation of thrombospondin promoter in Ni transformed cells (*13*). Since the Ni-transformed cells had an inactivated senescence gene and formed tumors in nude mice, it is possible that the senescence gene may participate as a transcriptional activator of the thrombospondin promoter.

The presence of high levels of thrombospondin has been shown to inhibit capillary growth towards the tumor cells, preventing tumor cells from growing in nude mice (i.e., antiangiogenesis) (*9*). Another study has demonstrated that overexpression of thrombospondin is associated with the ability of cells to grow in soft agar and to lose contact inhibition, but these cells could not form tumors in nude mice (*10*). Our work supports the importance of a low level of thrombospondin during Ni-induced malignant transformation.

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