Molecular Biology of Deregulated Gene Expression in Transformed C3H/10T1/2 Mouse Embryo Cell Lines Induced by Specific Insoluble Carcinogenic Nickel Compounds

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**In the past, exposure of workers to mixtures of soluble and insoluble nickel compounds by inhalation during nickel refining correlated with increased incidences of lung and nasal cancers. Insoluble nickel subsulfide and nickel oxide (NiO) are carcinogenic in animals by inhalation; soluble nickel sulfate is not. Particles of insoluble nickel compounds were phagocytized by C3H/10T1/2 mouse embryo cells and induced morphological transformation in these cells with the following order of potency: NiO (black) > NiO (green) > nickel subsulfide. Foci induced by black/green NiO and nickel monosulfide developed into anchorage-independent transformed cell lines. Random arbitrarily primed-polymerase chain reaction mRNA differential display showed that nine c-DNA fragments are differentially expressed between nontransformed and nickel compound–transformed 10T1/2 cell lines in 6% of total mRNA; 130 genes would be differentially expressed in 100% of the mRNA. Fragment R3-2 was a sequence in the mouse calnexin gene, fragment R3-1 a portion of the *Wdr1* gene, and fragment R2-4 a portion of the *ect-2* protooncogene. These three genes were overexpressed in transformed cell lines. Fragment R1-2 was 90% homologous to a fragment of the DRIP/TRAP-80 (vitamin D receptor interacting protein/thyroid hormone receptor–activating protein 80) genes and was expressed in nontransformed but not in nickel-transformed cell lines. Specific insoluble carcinogenic nickel compounds are phagocytized into 10T1/2 cells and likely generate oxygen radicals, which would cause mutations in protooncogenes, and chromosome breakage, and mutations in tumor suppressor genes, inactivating them. These compounds also induce methylation of promoters of tumor suppressor genes, inactivating them. This could lead to permanent overexpresssion of the *ect-2*, calnexin, and *Wdr1* genes and suppression of expression of the DRIP/TRAP-80 gene that we observed, which likely contribute to induction and maintenance of transformed phenotypes. *Key words:* clastogenesis, insoluble nickel compounds, morphological transformation, mRNA differential display, phagocytosis. *Environ Health Perspect* 110(suppl 5):845–850 (2002). *http://ehpnet1.niehs.nih.gov/docs/2002/suppl-5/845-850landolph/abstract.html*

In the past, occupational exposure of workers to mixtures of soluble and insoluble nickel compounds during specific nickel refinery operations correlated with increased incidences of nasal and respiratory cancer in workers (*1–6*). The U.S. National Toxicology Program (NTP) recently conducted rigorous animal carcinogenicity studies with specific insoluble and soluble nickel compounds to determine whether they were carcinogenic. The NTP studies showed that nickel subsulfide administered by inhalation induced lung tumors in F344 rats but not in B6C3F$_1$ mice (*7*). The NTP also showed that green (high temperature) NiO administered by inhalation caused lung tumors in rats, but evidence for tumorigenicity was equivocal in mice (*8*). Soluble nickel sulfate administered by inhalation was not carcinogenic to rats or mice (*9*).

Our laboratory (*10*) and the laboratory of M. Costa (*11–14*) have studied whether soluble and insoluble nickel compounds could induce morphological and neoplastic transformation in cultured mammalian cells. Our laboratory used a model *in vitro* cell culture system derived by Reznikoff et al. from embryos of C3H mice, designated C3H/10T1/2 Cl 8 (10T1/2) (*15*). This spontaneously immortalized, hyperdiploid cell line has a low saturation density, a low frequency of spontaneous morphological transformation, and a low frequency of spontaneous anchorage independence, and does not form tumors in nude mice (*15*). We and others showed that these cells undergo morphological transformation when exposed to a broad range of carcinogens, including polycyclic aromatic hydrocarbons (*16,17*), aromatic amine metabolites (*17*), radiation (*18*), aflatoxin B$_1$ (*19*), and lead chromate [(*20*); reviewed in *(21,22)*]. Many transformed cell lines derived from foci induced by chemical carcinogens and radiation in 10T1/2 cells grow in soft agar and form tumors when injected into nude mice [(*16–20;* reviewed in *(21,22)*].

Our laboratory showed that specific insoluble carcinogenic nickel compounds, including nickel subsulfide (*10,23*), crystalline nickel monosulfide (NiS) (*10*), black (low-temperature, LT) NiO (*23*), and green (high-temperature, HT) NiO (*10,23*), induce morphological transformation in 10T1/2 cells (Table 1) [reviewed in *(24–26)*]. We also showed that transformed cell lines derived from foci induced by crystalline NiS (*10*) and by green (HT) NiO (calcined at a temperature above 1,100°C) (*10, 27*) and black (LT) NiO (calcined at a temperature below 1,100°C) (*27*) have stable focus-forming and anchorage-independent phenotypes. One transformed cell line induced by green (HT) NiO forms fibrosarcomas when injected into nude mice (*10*). We are studying whether additional transformed cell lines induced by black (LT) NiO and by green (HT) NiO are tumorigenic (*27*).

Our findings that specific insoluble nickel compounds induce morphological transformation in 10T/12 cells show that 10T1/2 cells can be used as a model cell culture system to study cellular and molecular mechanisms of carcinogenesis induced by specific insoluble nickel compounds. These studies are relevant to understanding mechanisms by which nasal and lung cancers are induced in nickel refinery workers. Our laboratory has been conducting such studies. We also showed that concentrations of nickel subsulfide and green

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(HT) NiO that induce morphologic transformation in 10T1/2 cells do not induce mutation to ouabain resistance in these cells (Table 1) [(*10*); reviewed in (*24–26*)], and nickel subsulfide does not induce mutation to ouabain resistance or to 6-thioguanine resistance in cultured human diploid fibroblastic cells [(*28*); summarized in Table 1; reviewed in (*24–26*)]. These assays measure restricted types of base substitution mutations (ouabain resistance and 6-thioguanine resistance), frameshift mutations, and deletions (6-thioguanine resistance) but do not detect large multigenic deletions and mutations caused by oxygen radicals efficiently. Therefore, the mechanisms by which specific insoluble nickel compounds induce morphological and neoplastic transformation are unique.

In this article we describe and review our findings that specific insoluble nickel compounds induce cytotoxicity, micronuclei, chromosomal aberrations, and morphological transformation in 10T1/2 cells. We next describe and summarize our preliminary results, using random arbitrarily primed (RAP)-polymerase chain reaction (PCR) mRNA differential display to analyze gene expression in transformed 10T1/2 cell lines induced by crystalline NiS and green (HT) NiO and compare this to gene expression in nontransformed 10T1/2 cells. Finally, we synthesize our experimental findings to date into an overall model that delineates the molecular and cellular steps that occur during induction of morphological and neoplastic transformation of 10T1/2 mouse embryo cells by specific insoluble, carcinogenic nickel compounds.

Materials and Methods

Cells and cell culture. C3H/10T1/2 Cl 8 (10T1/2) mouse embryo cells were cultured in Basal Eagles Medium containing 10% fetal calf serum without antibiotics on Corning flasks and dishes (Corning Glass Co., Corning, NY, USA) as described (*10,15–17,21*). Samples of fetal calf serum obtained from suppliers (Omega Scientific, Inc., Tarzana, CA, USA; Gemini Bio-Products, Calabasas, CA, USA) were screened to identify those that supported a plating efficiency greater than 30%, a saturation density less than one million cells/60-mm cell culture dish, and 10–20 foci/20 dishes when cells were treated with 1 μg/mL of the carcinogen, 3-methylcholanthrene, or 3.75 μg/mL green (HT) NiO [(*10,17,20*); reviewed in (*21*)].

Phagocytosis of particles of insoluble nickel compounds. Phagocytic uptake of particles of insoluble nickel compounds was quantitated by our standard methods (*10*). Two hundred cells were seeded per 60-mm dish, five dishes per assay point, and treated 24 hr later with 25 μL of an acetone suspension containing the insoluble nickel compound to be studied in each 60-mm dish. Forty-eight hours later, medium was removed, and cells were rinsed with phosphate-buffered saline (PBS), fixed with 70% ethanol, then stained with crystal violet. Cells containing phagocytic vacuoles with one or more particles of insoluble nickel compounds were scored by light microscope (*10*). To determine whether phagocytosed particles were taken into lysosomes, cells were seeded, treated 24 hr later with acetone suspensions of insoluble nickel particles for 48 hr, stained in the living state with acridine orange, then examined by fluorescence microscopy (*23,29–31*). Cells containing particles of nickel compounds that colocalized with the red fluorescence of acridine orange in lysosomes were scored as containing particles taken up into lysosomes (*23*).

Cytotoxicity of insoluble nickel compounds. Cytotoxicity caused by insoluble carcinogenic nickel compounds was measured by our standard methods quantitating reduction in plating of treated cells (*10*). Cells were seeded, then treated 24 hr later with suspensions of insoluble nickel compounds in acetone for 48 hr. Ten days postseeding, or when colonies became visible by microscope, medium was removed from dishes, and cells were fixed with methanol, then stained with Giemsa. Colonies containing 20 or more cells were counted by dissecting microscope (*10,17,20,21*).

Chromosomal aberrations induced by insoluble nickel compounds. Cells were seeded, treated 24 hr later for 48 hr with suspensions of nickel compounds in acetone, treated with colcemid to induce metaphase arrest, swelled in hypotonic potassium chloride solution, then dropped onto slides. Slides were air dried, stained with Giemsa, and examined under a light microscope to determine the types and frequencies of chromosomal aberrations (*23,32,33*).

Induction of morphological transformation by insoluble nickel compounds. Morphological transformation was induced and quantitated by our standard methods [(*10,17,20*); reviewed in (*21*)]. Two thousand cells were seeded into each of twenty 60-mm dishes for each concentration of nickel compound tested, then treated 24 hr later with 25 μL acetone suspension of nickel compound in each 60-mm dish for 48 hr. Medium containing nickel compounds was removed and replaced with medium not containing nickel compounds, replaced twice per week until cells became confluent, then replaced once every week. Six weeks postseeding of assays, cells were rinsed with PBS, fixed with methanol, then stained with Giemsa. Type II and type III foci of morphologically transformed cells were scored with a dissecting microscope [(*10,17,20*); reviewed in (*21*)]. Transformed cell lines were developed from foci induced by green (HT) NiO and crystalline NiS and characterized as described (*10*).

Analysis of mRNAs of nontransformed and transformed 10T1/2 cell lines by RAP-PCR mRNA differential display. mRNA was extracted from nontransformed and transformed cell lines by standard methods (*34*) and copied into small complementary DNA (cDNA) fragments (100–500 base pairs [bp]) by reverse transcription (*35–37*). These small cDNA fragments were amplified by PCR, then analyzed on DNA sequencing gels (*35–37*). cDNA fragments present in nontransformed 10T1/2 cells but absent in one or more transformed 10T1/2 cell lines and fragments absent from nontransformed 10T1/2 cells but present in one or more transformed cell lines were eluted from gels, subcloned (*35–37*), and used as probes in Northern gel analyses (*34*). Subclones used as probes in Northern analysis that yielded the same pattern of differential gene expression seen in differential display gels were sequenced by automatic DNA sequencing. Sequences of fragments were entered into the BLAST database to determine homology with fragments of known genes (*38*).

Results

Phagocytosis of particles of insoluble nickel compounds by 10T1/2 cells and cytotoxicity of these particles to 10T1/2 cells. How do insoluble nickel compounds induce cytotoxicity in 10T1/2 cells? We found that

Table 1. Summary of the genotoxicity of specific insoluble nickel compounds.

	Ability to induce:					
Compound	Mutation to ouabain resistance in 10T1/2 cells	Mutation to ouabain resistance in diploid human fibroblasts	Mutation to 6-thioguanine resistance in diploid human fibroblasts	Micronuclei formulation in 10T1/2 cells	Chromosomal aberrations in 10T1/2 cells	Morphological transformation in 10T1/2 cells
Nickel subsulfide	–	–	–	+	+	+
Green (HT) NiO	–	ND	ND	+	+	+
Black (LT) NiO		ND	ND	+	+	+

Abbreviations: +, positive response, a dose-dependent and >2-fold increase; –, negative (no) response; ND, not determined. Data on mutagenicity of nickel subsulfide and green (HT) NiO in 10T1/2 cells from Miura et al. (*10*); data on mutagenicity of nickel subsulfide in diploid human fibroblasts from Biedermann and Landolph (*28*); data on morphological transformation from Miura et al. (*10*) and Verma et al. (*23*); data on induction of chromosomal aberrations from Verma et al. (*23*); data on micronuclei induction from Verma and Landolph (*39*). Some entries in this table are modified and adapted from Landolph (*24,25*).

10T1/2 cells actively phagocytose particles of certain insoluble nickel compounds (*10,23*). After a 48-hr treatment of 10T1/2 cells with particles of insoluble nickel subsulfide, cells exhibit one or more vacuoles with particles of insoluble nickel compounds (*10,23*). Phagocytosis of particles of insoluble nickel subsulfide is dependent upon the amount of particles added to cells and upon the length of time cells are treated with particles (*10,23*). Phagocytosis of particles of insoluble nickel subsulfide or crystalline NiS deposits a large amount of ionic nickel into cells (Figure 1) (*12,23*).

We next determined where inside cells the insoluble nickel compounds were deposited. In 10T1/2 cells treated with particles of nickel subsulfide, then stained in the living state with acridine orange, we observed the lysosomal network outlined by the orange fluorescence of acridine orange in the acidic environment of lysosomes by fluorescence microscopy. Fixing the same acridine orange–stained cells with 70% ethanol, staining them with crystal violet, and examining them by light microscopy demonstrated that particles of nickel subsulfide in vacuoles co-localized with the orange fluorescence of acridine orange in lysosomes (*23*). This indicated particles of nickel subsulfide were localized in lysosomes (*23*). In cells treated with black (LT) NiO or green (HT) NiO, the cellular location of these particles as visualized by light microscopy was the same as that of the orange fluorescence of acridine orange in the lysosomes (*23*). This indicated that particles of green and black NiO were also phagocytized and localized in lysosomes, although large phagocytic vacuoles were not apparent (*10,23*).

Uptake of particles of insoluble nickel compounds into cells generates intracellular nickel divalent (+2) ions, which bind to many proteins within cells, including proteins complexed to DNA (Figure 1). This is the first step in induction of cytotoxicity, chromosomal aberrations, and morphological transformation.

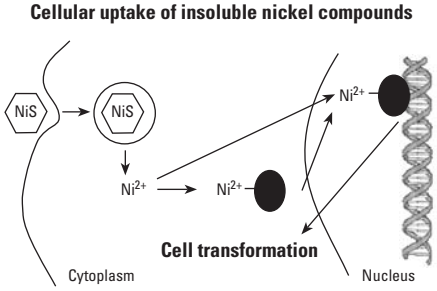

Figure 1. Summary figure and schematic figure detailing the ability of crystalline NiS and nickel subsulfide to be phagocytosed into mammalian cells, and the subsequent breakdown of the phagocytic vesicle, liberating Ni^{2+} ions into the cell, where they subsequently bind to proteins in the cell, including those proteins bound to DNA.

We showed that over concentration ranges at which particles of insoluble nickel compounds are phagocytosed, these particles induced dose-dependent cytotoxicity in 10T1/2 cells (*10,23*). The order of cytotoxicity we found for nickel compounds we studied was as follows: black (LT) NiO > green (HT) NiO > nickel subsulfide > soluble nickel sulfate (*10,23*).

Induction of chromosomal aberrations and morphological transformation in 10T1/2 cells by particles of insoluble nickel compounds. In 10T1/2 cells treated with particles of insoluble nickel compounds, after uptake of particles of insoluble nickel compounds by phagocytosis, we also observed chromosomal aberrations. Chromosomal aberrations induced in cells treated with insoluble nickel compounds included gaps, breaks, fragments, dicentrics, and satellite associations (*23*). Induction of chromosomal aberrations was dose dependent for each insoluble nickel compound used to treat 10T1/2 cells (*23*). The clastogenic potency of nickel compounds was green (HT) NiO > nickel subsulfide > black (LT) NiO > nickel sulfate (*23*).

We also showed that nickel subsulfide (*10,23*), green (HT) NiO (*23*), black (LT) NiO (*23*), and crystalline NiS (*10*) induced type II and type III foci of morphologically transformed cells in 10T1/2 cells (Table 1). The yield of type II + type III foci was dose dependent with each insoluble nickel compound studied (*10,23*). The efficiency with which these compounds induced morphological transformation was NiO (black) > NiO (green) > nickel subsulfide (*23*). When foci of transformed cells were cloned with glass cloning rings and expanded into transformed cell lines, the transformed cell lines stably maintained an ability to form foci and to form colonies in soft agar (*10,27*). One transformed cell line induced by green (HT) NiO formed progressively growing fibrosarcomas when injected into nude mice (*10*). Additional transformed cell lines derived from foci induced by treating 10T1/2 cells with black (LT) and green (HT) NiO are being tested for tumorigenicity. Therefore, 10T1/2 cells provide a cell culture system in which to study induction of morphological and neoplastic transformation of cells *in vitro* that can be used as a model for studying carcinogenesis by specific insoluble carcinogenic nickel compounds.

We found that nickel subsulfide and green (HT) NiO induced chromosomal aberrations (*23*), micronuclei (*39*), and morphological transformation (*10,23*) in 10T1/2 cells but did not induce mutation to ouabain resistance in 10T1/2 cells (Table 1) (*10*). Similarly, nickel subsulfide did not induce mutation to 6-thioguanine resistance and did not induce mutation to ouabain resistance in cultured diploid human fibroblasts (*28*) (Table 1).

Molecular biology of deregulation of gene expression in carcinogenic nickel compound–induced, transformed 10T1/2 cell lines. We next used the method of RAP–PCR mRNA differential display (*35–37*) to analyze gene expression in transformed cell lines induced by green (HT) NiO and crystalline NiS (*35–37*). When we analyzed 6% of the total mRNA of nontransformed and transformed cell lines by running five differential display gels, we found that nine genes were expressed differentially between nontransformed 10T1/2 cells and transformed cell lines induced by crystalline NiS and green (HT) NiO (*40*). Expression of some genes was decreased or extinguished, and some genes were overexpressed or their expression was activated in transformed 10T1/2 mouse embryo cell lines compared to their expression in nontransformed 10T1/2 cells (*40*). Extrapolation to 100% coverage of the mRNA indicated that approximately 130 genes are differentially expressed between nontransformed 10T1/2 cells and nickel compound–induced, transformed cell lines (*40*).

We isolated those 100–500 bp cDNA fragments that were differentially expressed from differential display gels, subcloned these fragments, then conducted Northern (*34*) or reverse Northern (*40*) blotting analysis with these subcloned fragments and RNA extracted from nontransformed and transformed 10T1/2 cells. We then selected those fragments whose expression in Northern or reverse Northern blot analysis reproduced the differential expression of the original fragments in the differential display gels. These fragments were sequenced automatically, and the sequences were entered into the BLAST database. We identified a number of RNA fragments that were overexpressed. One cDNA fragment was a fragment of the mouse calnexin gene (*40,41*), which maps to the q35 region of chromosome 5 of humans (*42,43*) and encodes a molecular chaperone (*44*) (Figure 2). A second cDNA fragment was a fragment of the *ect-2* gene (*45*), which maps to mouse chromosome band 3B and to 3q26.1-q26.2 in humans (*46,47*) and encodes a guanosine diphosphate (GDP)-guanosine triphosphate (GTP) exchange factor for the rho family of guanosine triphosphate (*48–50*). A third cDNA fragment was a fragment of the *Wdr-1* gene (*51*), a novel stress-response gene identified by the laboratory of M. Lomax (*52*) that maps to human chromosome 4p (*53*). One gene fragment whose expression is extinguished in transformed cell lines is a fragment of the DRIP/TRAP-80 gene (vitamin D receptor interacting protein/thyroid hormone receptor–activating protein 80) (*40*), which maps to chromosome 11 in humans (*54*). The protein product of the DRIP/TRAP-80 gene

serves as part of a complex that interacts with the vitamin D–vitamin D receptor complex to stimulate transcription (*55,56*) and with the thyroid hormone receptor–thyroid hormone complex to activate transcription (*57*). Additional genes whose expression is extinguished in the transformed cell lines include the insulin-like growth factor-1 receptor gene (*40*) and two unknown genes (*40*).

Discussion

Uptake of specific insoluble carcinogenic nickel compounds, genetic toxicology of these compounds, and molecular mechanisms of carcinogenesis induced by these compounds. Particles of specific insoluble carcinogenic nickel compounds, such as nickel subsulfide, crystalline NiS, green (HT) NiO, and black (LT) NiO, are taken into 10T1/2 cells and other cell types by phagocytosis (*10–12*) (Figure 1) and localize within lysosomes (*23*). Over the concentration ranges at which particles of insoluble nickel compounds were taken up into 10T1/2 cells, cytotoxicity (*10,23*), chromosomal aberrations (*23*), micronuclei (*39*), and morphological transformation (*10,23*) also occurred (Table 1). A plot of the slopes of the dose–response curves for morphological transformation induced by insoluble nickel compounds versus the percent of cells phagocytizing particles of insoluble nickel compounds was linear (*23*). Hence, uptake and internalization of particles of insoluble nickel compounds is an early and key step in morphological transformation induced by the specific insoluble nickel compounds we studied (*23*).

We showed that nickel subsulfide and NiO did not cause mutation to ouabain resistance in 10T1/2 cells (*10*), and nickel subsulfide did not cause mutation to ouabain resistance or mutation to 6-thioguanine resistance in diploid human fibroblasts (*28*). These two mutagenesis assays are not effective at detecting the specific types of mutations caused by oxygen radicals such as superoxide and hydroxyl radicals. For instance, adriamycin, which generates superoxide radicals [reviewed in *(58)*], does not elicit significant mutagenic responses in assays that detect mutation to ouabain resistance or mutation to 6-thioguanine resistance (*59*). We therefore propose alternative mechanisms other than the type of mutations detected in assays for ouabain resistance (specific base substitution mutations) or for 6-thioguanine resistance (base substitution and frameshift mutations and small deletions) to explain mechanisms of carcinogenicity of these specific insoluble nickel compounds. We propose first that cellular uptake of particles of insoluble nickel compounds is followed by intracellular solubilization of Ni^{2+} ions and their binding to cellular proteins, particularly those bound to DNA (Figure 1). We then propose that nickel ions bound to proteins that are bound to DNA (Figure 1), such as histones, react with intracellular hydrogen peroxide (H_2O_2) (Figure 3). The H_2O_2 would be generated by the action of superoxide dismutase on intracellularly generated superoxide (*60,61*). Ni^{2+} ions bound to proteins complexed to DNA could react with intracellular H_2O_2 in Fenton-like reactions (*60,61*). This would generate reactive hydroxyl radicals (Figure 3) [reviewed in (*60,61*)] that would then immediately react with DNA, leading to DNA damage, including 8-hydroxydeoxyguanosine, and mutations, which could induce cytotoxicity and chromosomal aberrations (Figure 3).

Hydroxyl radicals, generated by interaction of nickel ion–protein complexes proximate to DNA with H_2O_2 in Fenton-like reactions (Figure 3), likely induce mutations in protooncogenes, activating them into oncogenes (Figure 4), and likely also induce mutations in tumor suppressor genes, inactivating them. Ni^{2+} ion–generated hydroxyl radicals also likely inactivate tumor suppressor genes by causing loss or breakage of the chromosomes bearing these tumor suppressor genes (Figure 4). Therefore, Ni^{2+} ion–generated hydroxyl radicals likely cause some of the DNA damage observed in cells treated with insoluble nickel compounds. This DNA damage likely leads to cytotoxicity, micronuclei, chromosomal aberrations, and morphological transformation observed in cells treated with insoluble nickel compounds (Figures 3, 4) (*10–14,23–28*).

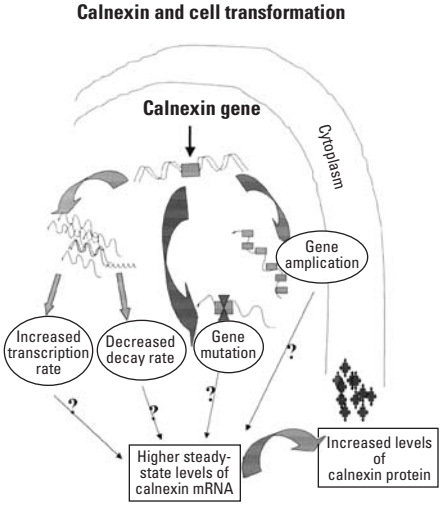

Figure 2. Schematic indicating that the mRNA and protein products of the calnexin gene are expressed at higher steady-state levels in transformed 10T1/2 cell lines induced by treatment of 10T1/2 cells with crystalline NiS and green (HT) NiO (*38,39*). Possible ways that could have caused the production of higher steady-state levels of calnexin mRNA and protein are suggested.

Deregulation of gene expression in nickel compound–transformed cell lines and implications for molecular mechanisms of carcinogenicity of insoluble nickel compounds. We now have some insight into the molecular mechanisms of morphological transformation induced by green (HT) NiO, black (LT) NiO, crystalline NiS, and nickel subsulfide in 10T1/2 cells. Nickel ion–generated H_2O_2 and hydroxyl radicals likely cause damage to approximately eight specific genes (Figures 3, 4). These events likely cause differential expression of approximately 130 genes between the nickel compound–induced, transformed 10T1/2 cell lines and nontransformed 10T1/2 cells that we observed (*40*). Four of these genes are likely protooncogenes. Activation of these protooncogenes into oncogenes then causes each one to stimulate signal transduction pathways downstream of these genes, such that each activated oncogene stimulates expression of approximately 16 genes (Figure 4). Four of these genes are probably tumor suppressor-like genes. Ni^{2+} ion–generated hydroxyl radicals cause mutation or chromosome breakage and inactivate these tumor suppressor genes (Figures 3, 4). These tumor suppressor–like genes may also become quiescent because of nickel ion–induced chromosomal condensation, resulting in methylation of the promoters of these genes (*29*). This results in the transcriptional silencing of approximately 64 genes controlled by these tumor suppressor–like genes. This model suggests that approximately 130 genes (8 original protooncogenes and tumor suppressor genes × 16 genes whose expression they each stimulate transcription of) would be differentially expressed in transformed cell lines induced by specific insoluble, carcinogenic nickel compounds (Figure 4). We predict that this combination of mutational activation of four protooncogenes into oncogenes and mutational inactivation, chromosomal breakage, or methylation of, and silencing of, four tumor suppressor–like genes, leads to deregulation of

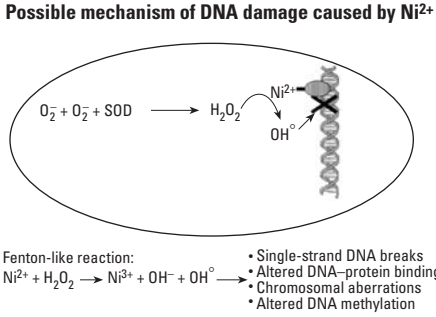

Figure 3. Schematic illustration of the generation of hydroxyl radicals in 10T1/2 cells by Ni^{2+} ion bound to proteins (histones) that are bound to DNA, and DNA damage that can be caused by this site-specific generation of hydroxyl radicals proximate to DNA.

the finely tuned gene expression of the normal cell. This stepwise deregulation of gene expression degrades the biological behavior of the nontransformed, well-regulated, contact-inhibited 10T1/2 cell, which cooperates with its neighboring cells, into that of a transformed, biologically autonomous cell. This is a stochastic process. For each tumor cell, a different set of genes may be altered, and they may be altered in a different order in time among tumor cells bearing the same changes in the same genes.

Which specific genes are targets for damage caused by nickel ion–generated, activated oxygen metabolites? How have these genes become damaged, and which of these damaged genes contribute to induction and maintenance of the transformed state? Our preliminary data indicate that one of these genes is the *ect-2* gene (*45*), which encodes a GDP–GTP exchange factor for the rho family of genes (*48–50*) and is located on human chromosome 3q26.1-q26.2 (*46*) and on mouse chromosome band 3B (*47*). This protooncogene can be activated in other systems to an oncogene by mutation (*48–50*). This gene is expressed at higher steady-state levels in transformed cell lines induced by crystalline NiS and green nickel (HT) oxide (*45*). We are working to determine whether and how this gene has become activated in transformed cell lines induced by these two insoluble nickel compounds (*45*). By precedent with other tumors bearing an activated *ect-2* oncogene, there may be mutations in the 5′ region of this gene, thus activating it.

A second gene we found expressed at higher steady-state levels in nickel compound–induced transformed cells is calnexin (Figure 2) (*40,41*). The calnexin gene has been localized to human chromosome 5q35 (*43*), and calnexin protein is a molecular chaperone (*44*). We are in the process of testing three hypotheses: *a*) that this gene is transcriptionally activated by other protooncogenes; *b*) that it acts as a protooncogene itself when mutated; or *c*) that overexpression of calnexin protein leads it to sequester and hence inhibit activity of proteins of tumor suppressor genes (Figures 2, 4).

A third gene overexpressed in transformed cell lines induced by NiS and green (HT) NiO is the *Wdr1* gene (*51*). The laboratory of M. Lomax first identified *Wdr1* as a novel gene overexpressed in the ears of chickens exposed to acoustic shock (*51*). *Wdr1* gene maps to human chromosome 4p (*53*). We do not know whether this gene functions as a protooncogene or whether its protein functions as a gene in a signal transduction pathway that can be stimulated by prior expression of an oncogene. Studies to answer these questions are in progress (*51*).

An intriguing gene that we found expressed in nontransformed 10T1/2 cells but not in nickel compound–induced, transformed 10T1/2 cell lines (*40*) encodes DRIP/TRAP-80 (*51–57*). The DRIP/TRAP-80 gene maps to human chromosome 11 (*54*). DRIP/TRAP-80 protein binds to the vitamin D–vitamin D receptor complex, conferring on this complex the ability to mediate transcriptional activation of specific genes by vitamin D (*55,56*). DRIP/TRAP-80 protein also helps activate the thyroid hormone–thyroid hormone receptor complex so it can mediate transcriptional activity conferred by thyroid hormone (*56*). We hypothesize that Ni^{2+} ions generate hydroxyl radicals (Figure 3), which cause inactivation of expression of the DRIP/TRAP-80 gene by causing inactivating mutations in this gene or by inducing breakage of the chromosome bearing this gene. Nickel ions themselves may bind to histones and cause chromosomal condensation (*29*), which results in methylation (*29*) of the promoter of the DRIP/TRAP-80 gene (Figures 3, 4). Loss of DRIP/TRAP-80 protein would abolish transcriptional activity of the vitamin D–vitamin D receptor complex, which is involved in tumor suppression (*54,55*). Studies are in progress in our laboratory to critically test these hypotheses of how the DRIP/TRAP-80 gene is inactivated.

The overall mechanistic model we propose to indicate how specific insoluble carcinogenic nickel compounds induce morphological and neoplastic transformation is as follows: *a*) These nickel compounds generate intracellular Ni^{2+} ions that induce oxygen radicals, which induce activating mutations in specific protooncogenes or protooncogene-controlled genes (*ect-2*, *Wdr-1*, and calnexin genes) and inactivating mutations or deletions in tumor suppressor or suppressor-like genes (DRIP/TRAP-80). *b*) Intracellular Ni^{2+} ions also cause chromosomal condensation and methylation of promoters of tumor suppressor genes, inhibiting expression of these genes and genes downstream that they transcriptionally control (*29*). These multiple events result in enhanced expression of approximately 64 genes whose expression is driven by the protein products of the *ect-2*, *Wdr1*, and calnexin genes and likely another protooncogene; loss of expression or significantly decreased expression of approximately 64 genes whose expression is driven by the DRIP/TRAP-80 protein and the DRIP/TRAP complex; and three other protein products of as yet unidentified genes (Figure 4). Accumulation of these events would severely degrade the growth control apparatus of the cell and convert the nontransformed cell into a transformed cell (Figure 4). Studies to critically test these hypotheses and this overall model are in progress in our laboratory.

Model for cell transformation induced by carcinogenic insoluble nickel compounds and methylcholanthrene

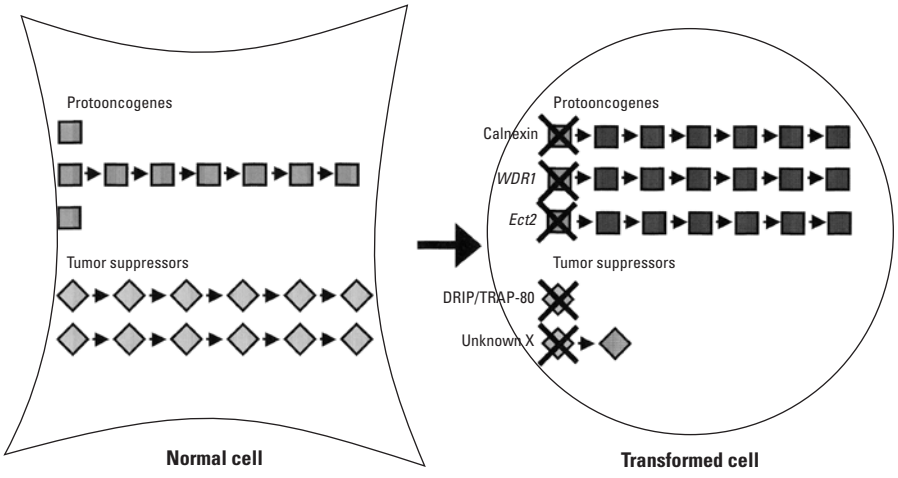

Figure 4. Schematic model for induction of morphological transformation in 10T1/2 cells treated with specific insoluble carcinogenic nickel compounds, such as nickel subsulfide, green (HT) NiO, black (LT) NiO, and crystalline NiS. These nickel compounds induce morphological transformation in 10T1/2 cells. They likely do so by generating hydroxyl radicals (Figure 3), which then cause mutation in and activation of protooncogenes into oncogenes. This causes induction of higher steady-state levels of calnexin mRNA, Wdr-1 mRNA, and ect-2 mRNA. These hydroxyl radicals also cause mutations in tumor suppressor genes and break chromosomes bearing tumor suppressor genes, leading to loss of and/or inactivation of, tumor suppressor genes. Intracellular nickel ions may also cause methylation of the promoters of tumor suppressor genes, leading to inhibition of their expression. This leads to inhibition of the synthesis of the mRNA encoded by the DRIP/TRAP-80 gene and by two novel unknown genes. Each activated oncogene can further activate expression of 20 other genes downstream of it and in the same signal transduction pathway. Inactivation of each tumor suppressor gene can also result in the loss of expression of approximately 20 other genes for which the protein product of this tumor suppressor gene acts as a transcriptional factor. These events in total can cumulatively result in the degradation of a nontransformed cell into a transformed cell.

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