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CHROMIUM EFFECTS ON CHONDROCYTIC DIFFERENTIATION IN VITRO

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A tissue culture study was conducted on the effects of chromium on chondrocytic differentiation. Mesenchymal cells from stage 22–24 chick limb buds were dispersed and cultured as micromasses, where they differentiated into chondrocytes. Addition of chromium(VI) to the cultures indicated that the production of proteoglycans (as detected by Alcian blue staining) was more sensitive to chromium's effects than was cell proliferation. Whereas Alcian blue nodule formation was inhibited by 1 μM Cr(VI), cell proliferation (as detected by cell counts) was not. Chromium (VI) was added to cultures at daily intervals, and these studies indicated that the interval of d 1–2 was the most sensitive period. ADP-ribose transferase activity in these cultures was measured; the pattern of enzyme activity in control cultures was high 1 and 24 h after the start of culture, decreased abruptly between 24 and 48 h, and then decreased more gradually. In the presence of Cr(VI), elevated ADP-ribose transferase levels were maintained throughout the culture period. We suggest that, in presence of 1.0 μM chromium(VI) or higher concentrations, the balance of events favors nucleolytic action rather than repair of damage.

INTRODUCTION

Tissue-culture techniques can be used to advantage in the study of chromium genotoxicity, especially in regard to chromosomal aberrations and cell proliferation. A review of the mutagenic and cytogenetic effects of chromium compounds by Levis and Bianchi (1982) points out the large number of studies carried out in tissue-culture systems. These studies indicate that the 6-valent form is responsible for the genotoxic effects observed in tissue culture systems.

Previously, we (Uyeki and Nishio, 1983) noted that in cultured Chinese hamster ovary (CHO) cells, the sister-chromatid exchange (SCE) effects of Cr(VI) were observed at 0.01–0.03 μM, whereas the antiproliferative effects were observed at 1.0–10.0 μM. Because of the 30- to 100-fold differences in effective concentrations, we suggested a working hypothesis to

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probe further into the cell biology of chromium effects: the genotoxic effects may be causally linked to the antiproliferative effects in tissue-culture systems.

A principal concern about genotoxicity is its close correlation with carcinogenicity (Norseth, 1981; Hayes, 1982). There is an implied notion of a reciprocal relationship between differentiation and carcinogenicity. On the one hand, cells differentiate and, as a result, stop proliferating. Conversely, in carcinogenicity, cells begin to proliferate and, as a result, are no longer differentiated. Hence, to experimentally evaluate the effects of chromium in differentiating tissues may give us insight into the cellular mechanisms of chromium carcinogenicity. The micromass culture technique of Solursh (Ahrens et al., 1977), using dispersed cells obtained from chick limb buds, has been used as a model to study cell differentiation; mesenchymal cells from stage 22-24 chick limb buds differentiate into chondrocytes. We used this assay system to determine whether chromium(VI) can affect mesenchymal cells to differentiate into chondrocytes. A criterion for the sensitivity of such a system is the effective molar concentration of chromium(VI)—that is, how it compares with chromium's effects on SCE or on cell proliferation.

METHOD

Chemicals

Benzamide (BAM) and nicotinamide adenine dinucleotide (NAD) were purchased from Sigma Chemical Co. (St. Louis, Mo.). [Adenine-2,8-^{3}H]NAD (25.0 Ci/mmol) was obtained from New England Nuclear (Boston, Mass.).

Cell Culture

Specific-pathogen-free (SPF) Cofal-negative eggs (Larson Lab-Vac Eggs Inc., Gowrie, Iowa) from White Leghorn hens were used as the source of wing and hind limb bud tissue. Embryonic states (23-24) were determined following the classification of Hamburger and Hamilton (1951). Limb bud cells were dissociated by enzymatic treatment with trypsin, collagenase, and DNase I (Nakanishi and Uyeki, 1985). Cells were washed twice with growth medium [Ham's F12 supplemented with 5% fetal calf serum (KC Biologicals Inc., Lenexa, Kans.) and penicillin/streptomycin] and resuspended at concentrations between 0.5×10^7/ml and 2.0×10^7/ml. One or four 20-μl drops cell suspension were placed in each well of a 12-well tissue-culture cluster dish (Costar, Cambridge, Mass.), and cells were allowed to attach to the substratum at 37°C for 90 min (Nakanishi and Uyeki, 1985). To each culture, 1 or 4 ml growth medium were added (with or without 3 m*M* BAM) and cultured for the indicated periods under a humidified atmosphere of 5% CO_2 in air at 37°C. Fresh growth medium (with or without BAM) was added in exchange for spent medium every day.

Cell Growth

A 20-μl drop of cell suspension was cultivated. Cells were harvested by trypsinization, suspended in Isoton counting fluid, and counted by using a Coulter counter and Channelyzer attached to a microcomputer for data collection and calculation (Uyeki and Nishio, 1983).

ADP-Ribose Transferase Activity in Permeabilized Cells

Four 20-μl drops of cell supsension were cultured in each well. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and treated with 0.4 ml cold permeabilizing buffer (which consisted of 10 m*M* Tris-HCl (pH = 7.8), 0.25 *M* sucrose, 1 m*M* EDTA, 4 m*M* $MgCl_2$, and 30 m*M* 2-mercaptoethanol) for 30 min in an ice bath (Nakanishi and Uyeki, 1985). A routine procedure of 40 triturations per well permeabilized greater than 95% of cells, as detected by Trypan blue staining. After permeabilization, the dish was transferred to a water bath at 25°C, and the enzyme assay was started by the addition of 0.2 ml substrate mixture consisting of 100 m*M* Tris-HCl (pH = 7.8), 30 m*M* $MgCl_2$ and 3 μ*M* [^{3}H]NAD (90,000 cpm/nmol). The reaction was continued for 30 min and then terminated by adding 1.0 ml of 20% cold trichloracetic acid (TCA) and chilling in ice. The radioactivity in the acid-insoluble fraction was determined as reported previously (Uyeki et al., 1976).

Analysis of Alcian Blue-Stained Nodules

Alcian blue stain was used to identify acidic proteoglycans when tissues were stained at pH 1.0. After the culture period, media were removed and the following sequence was used to prepare the nodules for staining: 30 min of 95% ethanol, 30 min of 10% formalin, and 60 min of 1% Alcian blue staining. The supernatant containing Alcian blue was removed by decantation and, to remove excess Alcian blue, washed 3 times with distilled water and once with 3% acetic acid. Alcian blue-stained nodules in a well were then counted. For the spectrophotometric analysis of Alcian blue associated with proteoglycans of the nodules, 1.0 ml of 4.0 *M* guanidine was added and left in the wells overnight at 4°C to extract the Alcian blue from the nodules (Hassell and Horigan, 1982). Subsequently, the guanidine solution was analyzed for Alcian blue on a spectrophotometer using 620-nm wavelength to measure the optical density of the color.

Statistics

The data were analyzed by Duncan's test, following an analysis of variance. The level of significance chosen was $p = 0.01$.

RESULTS

Micromass cultures of chick limb bud mesenchymal cells were plated so that 20 μl contained 400,000 cells. After 90 min of cultivation, culture

media and chromium(VI) were added to the cultures. After 4 d of culture, cells were analyzed for the presence of Alcian blue-stained nodules and for cell counts. The results of this study are shown in Table 1. The results indicated that the addition of chromium(VI) is without effect, except at a concentration of 1.0 μM. In this case, the numbers of Alcian blue-containing nodules were significantly reduced to values that were 58% of control values. We note that at 1.0 μM cell counts did not differ significantly from control values, indicating that cell proliferation was not significantly affected.

To determine the effect of daily pulses of chromium(VI) on cartilage nodule formation of mesenchymal cells in vitro, chromium(VI) was added to cultures daily and remained in contact with cultured cells for 24 h, after which the culture media were removed and new media added. Controls without chromium were also run for each day, and the media were removed and new media added. This procedure was used for each subsequent day. The results of these experiments are shown in Table 2. The results indicate that the sensitive period of chondrocytic differentiation is between d 1 and 2 of incubation. At d 1-2, the chondrocytic nodules were reduced to levels that were 45% of control values. At no other 24-h period of time was chromium(VI) as effective in inhibiting the ability of mesenchymal cells to differentiate into chondrocytes.

To assess whether or not the production of Alcian blue-staining proteoglycans was more sensitive to chromium's action than was cell proliferation, the following experiment was performed. Twenty-microliter droplets of cell suspension (containing 400,000 cells) were cultured as micromasses for 4 d. Chromium(VI) was added on d 1 of incubation and left for the duration of the culture period. After 4 d of culture, the cultures were terminated and extracted for Alcian blue according to procedures described in the method section. The results are shown in Table 3. They indicated that the Alcian blue extracted from the cartilage nodules was significantly

TABLE 1. Effects of Cr(VI) on Cartilage Nodule Formation and Cell Growth of Stage 22-24 Chick Limb Bud Cell Cultures[a]

Cr(VI) concentration	Number of nodules	Percent of control	Cell counts ($\times 10^5$)	Percent of control
Control	201	–	5.96	–
0.1 μM	218	108	5.52	93
0.3 μM	214	106	5.52	93
1 μM	116	58[b]	5.60	94

[a]A 20-μl drop of cell suspension (2×10^7 cells/ml) was cultured as micromass for 4 d in the presence of chromium (VI). Six cultures were run for each concentration of drug; triplicates were used for Coulter cell counts, and triplicates were used for scoring Alcian blue-stained nodules.

[b]Significantly different at the 0.01 level of significance. Statistical analysis was Duncan's test following an analysis of variance.

TABLE 2. Effects of Daily Pulses of 1.0 μM Cr(VI) on Cartilage Nodule Formation of Stage 22-24 Chick Limb Bud Cell Cultures[a]

Day of pulse	Nodules per culture (mean ± SE)	Number of experiments	Total number of replicates	Percent of control
Day 0-1				
Control	165 ± 14	3	8	–
Chromium	145 ± 29	3	8	88
Day 1-2				
Control	213 ± 18	4	10	–
Chromium	97 ± 17	4	10	45[b]
Day 2-3				
Control	146 ± 26	3	9	–
Chromium	130 ± 18	3	9	89
Day 3-4				
Control	64 ± 12	1	3	–
Chromium	77 ± 6	1	3	120

[a]A 20-μl drop of cell suspension (2 × 10^7 cells/ml) was cultured as micromass for 4 d. Chromium was added at indicated daily intervals; after 24 h the media were removed, cultures washed once, and fresh media added.

[b]Significantly different from its control value at the 0.01 level of significance, using Duncan's test.

lower than untreated controls; the addition of 0.3 and 1.0 μM Cr(VI) on d 1 of culture significantly reduced the extractable Alcian blue to values that were 55 and 22% of controls, respectively. Hence, the products of the differentiated chondrocyte (proteoglycans represented by the extracted Alcian blue) were more sensitive to chromium's action than was cell proliferation.

Previously (Nishio et al., 1983; Nakanishi et al., 1984), we reported that inhibitors of ADP-ribose transferase—namely, benzamide and nicotinamide—

TABLE 3. Effects of Cr(VI) on Alcian Blue Stain Extracted from Cartilage Nodules Formed in Stage 22-24 Chick Limb Bud Cell Cultures

Cr(VI) concentration	Number of replicate cultures	Extracted Alcian blue (mean ± SE)	Percent of control
Control	7	2.23 ± 0.01	–
0.1 μM	4	1.84 ± 0.26	82
0.3 μM	4	1.23 ± 0.19	55[b]
1 μM	8	0.50 ± 0.02	22[b]

[a]A 20-μl drop of cell suspension (2 × 10^7 cells/ml) was cultured as micromass for 4 d. Chromium(VI) was added on d 1 of incubation for the duration of the culture period. The Alcian blue extracted from the proteoglycan of cartilage nodules is expressed in terms of microgram equivalent of Alcian blue.

[b]Significantly different from controls at the 0.01 level of significance. Statistical analysis was Duncan's test following an analysis of variance.

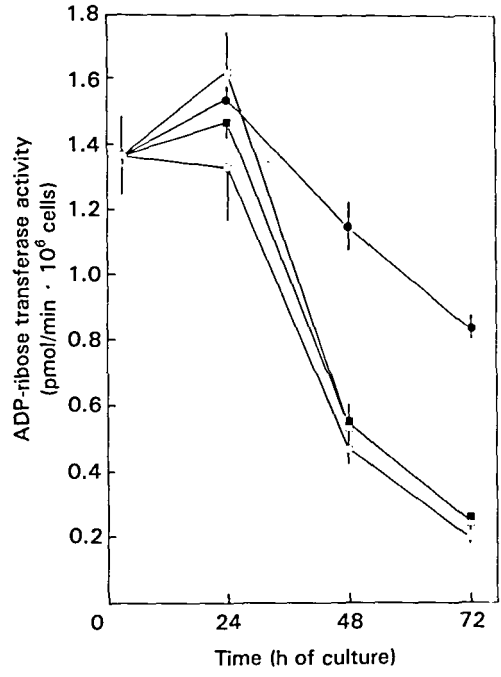

FIGURE 1. ADP-ribose transferase activity in chick limb bud cell cultures. Four 20-μl drops of cell suspension (2 X 10^7/ml) were cultured as micromass in the presence of chromium (1 μ*M*) and/or ascorbic acid. Each point is the mean of triplicate cultures; the bars show the standard deviation (SD); open circles, untreated cultures; dark circles, chromium-treated cultures; open squares, ascorbic acid-treated cultures; dark squares, chromium- and ascorbic acid-treated cultures.

enhanced chondrocytic differentiation and suggested that poly(ADP-ribosyl)ation may be a regulatory mechanism in cell differentiation of these mesenchymal cells. We studied the influence of chromium(VI) on ADP-ribose transferase (ADPRT) activity. These results are shown in Fig. 1. The enzyme activity in untreated cultures (open circles) was high at 1.5 and 24 h after the start of culture, decreased sharply between 24 and 48 h, and less so at 72 h after the start of culture. On the other hand, in chromium(VI)-treated cultures (dark circles), the enzyme activity persisted at higher levels. At 72 h after the start of culture, the ADPRT activity of control cultures was 14% of 1.5-h values (the starting point of all cultures), whereas in chromium-treated cultures, the values were 57% of 1.5-h values; hence, the ADPRT activity persisted at a higher level in chromium-treated cultures. We had previously shown that ascorbic acid (50 μg/ml) was able to prevent chromium toxicity in CHO cells in vitro (Uyeki and Nishio, 1983). Similarly, ADPRT activity in chromium-treated cells with ascorbic acid (dark squares) approached values that were similar to untreated cultures or cultures with ascorbic acid alone (open squares).

DISCUSSION

Many DNA-damaging agents such as genotoxicants, anticancer agents, mutagens, or carcinogens increase SCE frequencies in vertebrate cells. Chromium is one of these agents, and we have shown that enhanced SCE frequencies occur at 10–30 n*M* Cr(VI) in cultured CHO cells. Based mainly on the effective molar concentrations of Cr(VI) (0.01 μM for enhanced SCE effects and 1.0 μM for inhibition of cell proliferation), we suggested that the SCE effects may be causally linked to the antiproliferative effects. In order to probe the heuristic value of this notion, we selected the differentiation of chondrocytes from chick limb bud mesenchymal cells as a suitable test system. We (Nakanishi and Uyeki, 1985) earlier showed that when stage 22–25 chick limb bud cells are cultured for 96 h, they undergo the following sequence: activation of ADP-ribose transferase, DNA synthesis, cell proliferation, nodule formation, and proteoglycan synthesis (detected by Alcian blue staining and radioactive sulfur incorporation).

Addition of chromium(VI) to cultures of mesenchymal cells from the chick limb bud indicated that the products of chondrocytic differentiation, that is, the proteoglycans (as detected by Alcian blue staining), are more sensitive to the noxious effects of chromium than is cell proliferation. The results are shown in Tables 1–3. Whereas 1.0 μM and lower concentrations of chromium(VI) did not significantly alter cell proliferation (as depicted in Table 1 by the lack of significant differences in cell counts), the number of Alcian blue-staining nodules decreased significantly in cultures treated with 1.0 μM chromium(VI). At concentrations lower than 1.0 μM (that is, 0.3 and 0.1 μM), Alcian blue-staining nodules were not affected.

The difference in chromium sensitivity between cell proliferation and cell differentiation is even more evident in the experiments dealing with extractable Alcian blue from chondrocytic nodules. This was shown in Table 3. Thus, the Alcian blue extracted from cultures treated with 0.3 and 1.0 μM Cr(VI) was 55 and 22% of untreated control cultures, respectively. The studies depicted in Tables 1 and 3 corroborate the notion that cell differentiation (experimentally defined here as extractable Alcian blue) is more sensitive to the inhibitory effects of Cr(VI) than is cell proliferation of the mesenchymal cells. Although other alternatives have not been excluded, an explanation with current appeal for this enhanced chromium sensitivity is the complexity of cell differentiation; thus, a complex event such as chondrocytic differentiation, which requires a successful completion of several coordinated events within a circumscribed time frame, will be more sensitive to chromium's action than one of its component events (such as cell proliferation).

One characteristic of cell differentiation that we can experimentally examine is the temporal dependency of events. We posed a question: Is there a time period when the differentiation of mesenchymal cells to

chondrocytes is most sensitive to chromium's action? Results, shown in Table 2, indicated that chondrocytic differentiation is most sensitive to 1.0 μM chromium's action between d 1 and 2 of culture, inhibiting the production of Alcian blue-staining nodules to values that were 45% of controls. The 24-h pulses of chromium, before or after this time period, were ineffective in altering chondrocytic differentiation. Thus, the critical time period for chromium's actions is d 1–2 of cultivation. Further speculation about the reasons for this time dependency is unprofitable, since additional experiments are required. This work is continuing.

Previously, we demonstrated that ADP-ribose transferase activity in mesenchymal cells, as micromass cultures, is high. The activity of such cultures is high at 1 and 24 h after the start of culture, decreases abruptly between 24 and 48 h, and then decreases more gradually. This was shown in control cultures in Fig. 1. On the one hand, the addition of 1.0 μM Cr(VI) to the cultures maintained elevated ADP-ribose transferase at levels that were significantly above control levels at 72 h. We interpreted these results to mean that, in the continued presence of a genotoxicant such as chromium, there is continued DNA damage, which leads to continued elevated activity of ADP-ribose transferase activity. On the other hand, other cultures, either with vitamin C alone or those in which vitamin C was added to prevent chromium effects (Uyeki and Nishio, 1983), were not significantly different from the pattern of enzyme activity observed in control cultures.

Our results on chromium(VI) genotoxicity to the limb bud cultures are generally in accord with the notions of Cleaver and Morgan (1985), who recently suggested a hypothesis for the role of poly(ADP-ribose) synthesis in damaged cells: DNA strand breaks set in motion a cellular response that results, among other events, in the activation of nucleases. Poly(ADP-ribosyl)ation is also a cellular response to DNA strand breaks by which a large array of proteins becomes covalently modified for a brief period. The turnover of ADP-ribosyl groups occurs in a matter of minutes. In those instances where enzyme activity has been directly measured, ADP-ribosylation inhibits enzyme activity. Poly(ADP-ribosyl)ation inhibits enzymes (such as the nucleases) that are activated promptly by alkylating agents and other genotoxicants. Thus, it appears that there are normal feedback mechanisms in the cell to limit the degree of damage; for instance, whereas DNA strand breaks set in motion a chain of nucleolytic events, one of which is the activation of nucleases, other events, such as poly-(ADP-ribosyl)ation, by covalently attaching to these chromatin proteins, exert a damage-limiting influence. In the presence of 1.0 μM or higher concentrations of Cr(VI), DNA strands continue to break, favoring the balance of events toward nucleolytic action rather than repair of damage.

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