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Benzamide on chondrocytic differentiation in chick limb bud cell culture

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SUMMARY

Benzamide, an inhibitor of (ADP-ribose) transferase, augmented chondrocytic differentiation of chick limb bud mesenchymal cells in micromass cultures; the incorporation of $^{35}SO_4^{2-}$ into the trichloroacetic-acid-insoluble constituents of cell masses as well as the formation of cartilage nodules (Nishio, Nakanishi, Doull & Uyeki, 1983) occurred about 24 h earlier than in untreated cultures and continued to be enhanced in benzamide-treated cultures of stage 23- to 24-chick limb bud cells. Benzamide also significantly increased cell proliferation. However, benzamide did not affect DNA and RNA syntheses except for one period: 24 to 30 h after the start of culture, RNA synthesis was stimulated. From 48 h of culture, (ADP-ribose) transferase activity decreased daily in untreated cultures, whereas benzamide treatment diminished (ADP-ribose) transferase activity 24 h earlier. On the other hand, intracellular NAD levels increased daily in untreated cultures, and benzamide significantly increased the NAD levels above untreated cultures. ATP levels did not differ significantly during the culture period, and benzamide did not affect ATP levels.

INTRODUCTION

The relationship between cell differentiation, NAD and poly (ADP-ribose), occupies the attention of many workers (Terada, Fujiki, Marks & Sugimara, 1979; Farzaneh, Zalin, Brill & Shall, 1982; Kanai *et al.* 1982; Morioka, Tanaka & Ono, 1982*a*; Morioka, Tanaka, Ishizawa & Ono, 1982*b*; Pekala & Moss, 1983). NAD is involved in chondrocytic differentiation (Caplan, 1972; Rosenberg & Caplan, 1974; Rosenberg & Caplan, 1975) and there may be a correlation between intracellular poly (ADP-ribose) levels or synthesis and the early phases of cell differentiation and development of chick limb bud mesenchyme (Caplan & Rosenberg, 1975; Caplan, Niedergang, Okazaki & Mandel, 1979). We reported previously that inhibitors of (ADP-ribose) transferase, namely nicotinamide, benzamide (BAM) and its analogs, enhanced chondrocytic differentiation in cell cultures of chick limb bud and suggested that poly ADP-ribosylation may be a regulatory mechanism in cell differentiation (Nishio *et al.* 1983).

Key words: Chondrocytes, differentiation, proliferation, (ADP-ribose) transferase, nicotinamide adenine dinucleotide.

In order to understand the mechanisms whereby inhibitors of (ADP-ribose) transferase augment chondrocytic differentiation, we studied the changes in cell growth, sulphated proteoglycan synthesis, DNA synthesis, RNA synthesis, (ADP-ribose) transferase activity, and intracellular NAD and ATP in cultured chick limb bud mesenchymal cells.

MATERIALS AND METHODS

Chemicals

Benzamide (BAM), nicotinamide adenine dinucleotide (NAD), adenosine 5′-triphosphate (ATP) and Alcian blue were purchased from Sigma Chemical Co. (St. Louis, MO). $^{35}SO_4^{2-}$ (carrier-free, 43 Ci/mg) was obtained from ICN Pharmaceuticals (Irvine, CA). [2-^{14}C]thymidine (54·0 mCi/mmol) was obtained from Schwarz/Mann (Spring Valley, NY). [6-^{3}H]uridine (22·4 Ci/mmol) and [adenine-2,8-^{3}H]NAD (25·0 Ci/mmol) were obtained from New England Nuclear (Boston, MA).

Cell culture

SPF Cofal negative eggs (Larson Lab-Vac Eggs Inc., Gowrie, IA) from white Leghorn hens were used for the source of wing and hind limb bud tissue. Embryonic stages (23–24) were determined following the classification of Hamburger & Hamilton (1951). Limb bud cells were dissociated by enzymatic treatment with trypsin, collagenase and DNase I, as reported previously (Nishio *et al.* 1983). Cells were washed twice with growth medium [Ham's F12 supplemented with 10% foetal calf serum (KC Biologicals Inc., Lenexa, KS) and penicillin/streptomycin] and resuspended at a concentration of twenty million cells per ml. 20 to 200 μl of cell suspension were placed in the centre of each well of a 12-place tissue culture cluster dish (Costar, Cambridge, MA), and cells were allowed to attach to the substratum at 37 °C for 90 min (Ahrens, Solursh & Reiter, 1977); in this manner, microdroplets of cell suspension formed foci of densely packed cells. To each culture 1 to 5 ml of growth medium were added (with or without 3 mM-BAM) and cultured for the indicated periods under a humidified atmosphere of 5% CO_2 in air at 37 °C. Fresh growth medium was added in exchange for spent medium at 1 ml a day per 1×10^6 inoculated cells.

Cartilage nodule formation

Micromass foci, produced from 20 μl of cell suspension, were cultured for 2 to 5 days. Cells were fixed with 95% ethanol and 10% formalin, and stained with 1% Alcian blue at pH 1 to stain sulphated glycosaminoglycans in a cartilage matrix (Lev & Spicer, 1964). This assay was performed to insure that suitable differentiation and proliferation had occurred.

Cell growth

A 20 μl drop of cell suspension was cultured. Cells were harvested by trypsinization, suspended in Isoton counting fluid, and counted by using a Coulter Counter and a Channelyzer attached to a microcomputer for data collection and calculation. The distribution of cell size in BAM-treated cultures did not differ from that in untreated cultures.

Sulphated proteoglycan, DNA and RNA syntheses

A 30 μl drop of cell suspension was cultured. $^{35}SO_4^{2-}$ or [^{14}C]thymidine and [^{3}H]uridine were added to cultures at concentrations of 10, 0·5 and 2 μCi/ml, respectively, and cells were cultured for an additional 6 h. To determine the incorporation of $^{35}SO_4^{2-}$ into the cell mass, cells were

washed twice with ice-cold phosphate-buffered saline (PBS), and 1 ml of cold 10 % trichloroacetic acid (TCA) was added to each well. TCA was discarded, and the cell precipitate was placed on Whatman chromatography paper and washed with 5 ml of 5 % TCA and 3 ml of ethanol (Uyeki, Truitt & Bisel, 1976). To determine DNA and RNA syntheses, cells were harvested by trypsinization, washed twice with cold PBS, resuspended in cold 10 % TCA, placed on the chromatography paper and prepared for scintillation counting as mentioned above. The radioactivity in the acid-insoluble fraction was determined in 6 ml of xylene-based scintillation counting fluid with a Packard Tri-Carb Model 3320 liquid scintillation spectrometer.

(ADP-ribose) transferase activity in permeabilized cells

Four 20 μl foci of cell suspension were cultured in each well. Cells were washed twice with cold PBS and treated with 0·4 ml of cold permeabilizing buffer (which consisted of 10 mM-Tris-HCl (pH = 7·8), 0·25 M-sucrose, 1 mM-EDTA, 4 mM-$MgCl_2$ and 30 mM-2-mercaptoethanol) for 30 min in an ice bath (Berger & Johnson, 1976; Berger, Weber & Kaichi, 1978; Farzaneh *et al.* 1982); a routine procedure of 40 pipettings per well permeabilized greater than 95 % of cells to trypan blue staining. After permeabilization, the dish was transferred to a water bath at 25 °C, and the (ADP-ribose) transferase assay was started with the addition of 0·2 ml of substrate mixture consisting of 100 mM-Tris-HCl (pH = 7·8), 30 mM-$MgCl_2$ and 3 μM-[^{3}H]NAD (90 000 c.p.m./nmol). The reaction was continued for 30 min, and terminated by adding 1·0 ml of 20 % cold TCA, and chilled in ice for 30 min. The radioactivity in the acid-insoluble fraction was determined as mentioned above.

Nucleotide levels

A 200 μl drop of cell suspension was cultured. Cells were washed twice with cold PBS, and 100 μl of cold 5 % perchloric acid (PCA) were added to each well and kept in an ice bath for 5 min. The supernatant obtained from three wells was then put into a 1·5 ml microcentrifuge tube and neutralized with cold KOH (Wehr, 1980; Nissinen, 1980; Zakaria & Brown, 1981). The potassium perchlorate precipitate was removed by centrifugation, and the near neutral (pH = 5·5–6·0) aqueous solution of nucleotides was filtered through a 0·22 μm microfilter. This direct extraction method was quicker and required less manipulation of cells, and nucleotide values were higher and more reproducible than with the conventional indirect extraction method which requires trypsinization and centrifugation before extraction.

High-performance liquid chromatography (HPLC) analysis was performed on a Gilson liquid chromatography system equipped with a variable wavelength u.v. detector (set at 254 nm). The separations described here were performed on a 4·6×250 mm Partisil 10-SAX column (Chromanetics, Jessup, MA) at an eluent flow rate of 4 ml/min. The low-concentration buffer was 7 mM-KH_2PO_4 (pH = 4·0), and the high concentration buffer was 250 mM-KH_2PO_4 (pH = 4·5) containing 500 mM-KCl. 200 μl of the extract obtained from cultured cells were injected. After an isocratic period of 8 min with the low-concentration buffer, a linear gradient with the high-concentration buffer was applied for 40 min; finally, an isocratic period of 15 min with the high-concentration buffer was used. The software of this elution protocol was stored on 5·25 inch floppy disks of the Gilson controller. The column was regenerated by washing with 60 ml of the low-concentration buffer before the next analysis.

A standard solution containing known amounts of nucleotides was chromatographed prior to the analysis. The area under each peak on the recorder of the u.v. detector was used for calculation of nucleotide concentrations in the samples (Wehr, 1980). Also, to measure DNA in cultured cells, a modification of the method of Schmidt-Thannhauser-Schneider (STS) was used (Schneider, 1945; Schneider, 1946; Mizuno, 1977), and nucleotide content was expressed in terms of DNA content.

Statistics

The data were analysed by Duncan's test, following an analysis of variance. The level of significance chosen was $P = 0·01$.

RESULTS

Cell growth (Fig. 1)

At 24 h after the start of culture, the number of cells was $(2{\cdot}78 \pm 0{\cdot}24) \times 10^5$ in untreated cultures and $(3{\cdot}21 \pm 0{\cdot}33) \times 10^5$ in BAM-treated cultures; approximately 30 % and 20 % of inoculated cells were lost during this period, respectively. In untreated cultures, cell proliferation was observed after 48 h; in BAM-treated cultures, cell proliferation was observed after 24 h. Subsequently, the numbers of cells continued to increase in both groups and the growth curve of BAM-treated cultures had a slightly steeper slope than in untreated cultures. Using the criteria of cell counts, cell size and cell viability, 3 mM-BAM was not toxic to our cultures.

Sulphated proteoglycan synthesis (Fig. 2)

The sulphated proteoglycan synthesis in cultured cells was shown by the incorporation of $^{35}SO_4^{2-}$ into the acid-insoluble fraction of cells. There were no

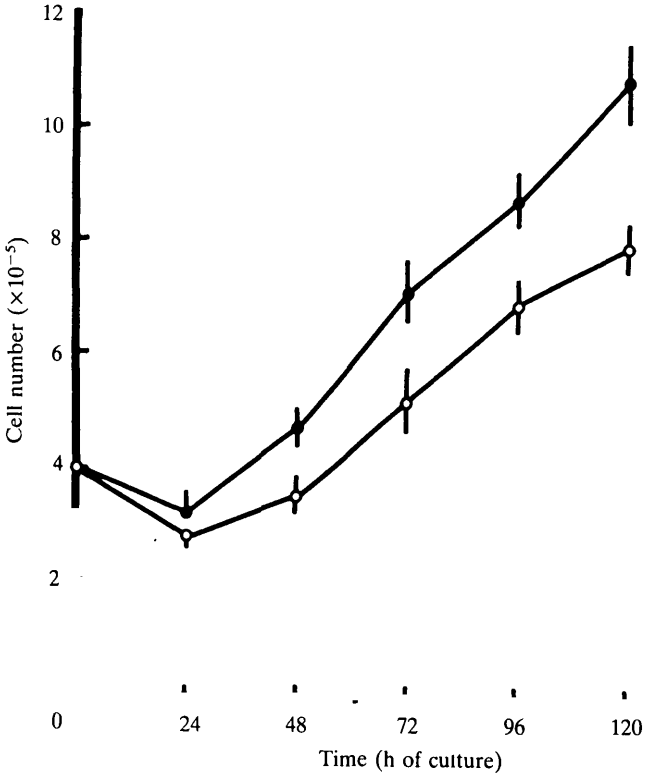

Fig. 1. Cell growth in chick limb bud cell cultures. A 20 μl drop of cell suspension (2×10^7/ml) was cultured. Cells were harvested with trypsin treatment, and the number of cells was counted by using a Coulter Counter. Each point is the mean of three independent experiments of triplicate cultures; the bars represent the standard deviation of the mean; ○, untreated cultures; ●, BAM-treated cultures.

significant increases in either untreated and BAM-treated cultures until day 3 of culture (assay done from 48 to 54 h) when nodule formation occurred in BAM-treated cultures; nodule formation in untreated cultures was not observed until 1 day later (also reported in Nishio *et al.* 1983). After that, however, the incorporation of $^{35}SO_4^{2-}$ in BAM-treated cultures increased exponentially, whereas the slope in untreated cultures was more gradual. The incorporation of $^{35}SO_4^{2-}$ occurred later in untreated cultures than in BAM-treated cultures.

DNA and RNA syntheses (Fig. 3)

DNA and RNA syntheses were shown by the incorporation of [^{14}C]thymidine and [^{3}H]uridine into the acid-insoluble fraction of cultured cells, respectively. DNA synthesis showed the lowest value on day 1 (0 to 6 h) in both groups and reached a peak value on day 3 (48 to 54 h) in untreated cultures; on the other hand,

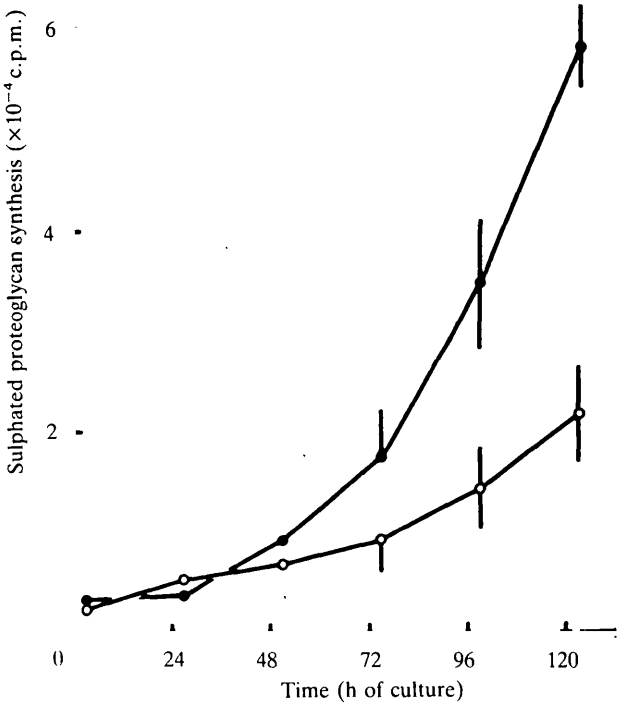

Fig. 2. Sulphated proteoglycan synthesis in chick limb bud cell cultures. A 30 μl drop of cell suspension (2×10^7/ml) was cultured. $^{35}SO_4^{2-}$ was added at the concentration of 10 μCi/ml, and cells were cultured for an additional 6 h. The sulphated proteoglycan synthesis was shown by the incorporation of $^{35}SO_4^{2-}$ into the acid-insoluble fraction of the cell mass. Each point is the mean of six cultures; the bars represent the standard deviation of the mean; ○, untreated cultures; ●, BAM-treated cultures.

BAM-treated cultures reached a peak value earlier on days 2–3. However, there were no significant differences between the two groups. RNA synthesis in both groups exhibited a biphasic curve; thus, an increase was observed until 54 h of culture, followed by an abrupt decrease during the next 24 h and, subsequently, followed by a gradual increase.

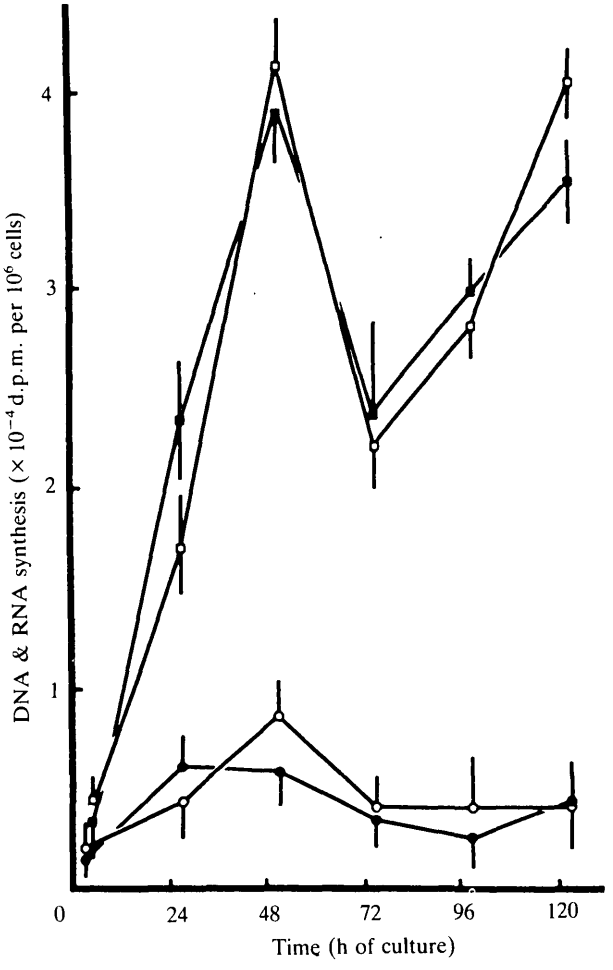

Fig. 3. DNA and RNA syntheses in chick limb bud cell cultures. A 30 μl drop of cell suspension (2×10^7/ml) was cultured. [^{14}C]thymidine and [^{3}H]uridine were added at the concentration of 0·5 and 2 μCi/ml, and cells were cultured for an additional 6 h. DNA and RNA syntheses were shown by the incorporation of [^{14}C]thymidine and [^{3}H]uridine into the acid-insoluble fraction of 1×10^6 cells, respectively. Each point is the mean of six cultures; the bars represent the standard deviation of the mean; ○, DNA synthesis in untreated cultures; ●, DNA synthesis in BAM-treated cultures; □, RNA synthesis in untreated cultures; ■, RNA synthesis in BAM-treated cultures.

(ADP-ribose) transferase activity in permeabilized cells (Fig. 4)

The activity in untreated cultures was high at 1 h and 24 h after the start of culture, decreased abruptly between 24 h and 48 h, and then diminished gradually with time. The activity in BAM-treated cultures showed no significant difference at 1 h compared with that in untreated cultures, but after that an abrupt decrease was observed until 48 h, and then diminished gradually; BAM pretreatment of intact cultures significantly decreased the activity from 24 h compared with untreated cultures. Since the activity in BAM-treated cultures did not differ from that in untreated cultures at 1 h, the possible presence of residual BAM was not considered significant when the enzyme assay was done.

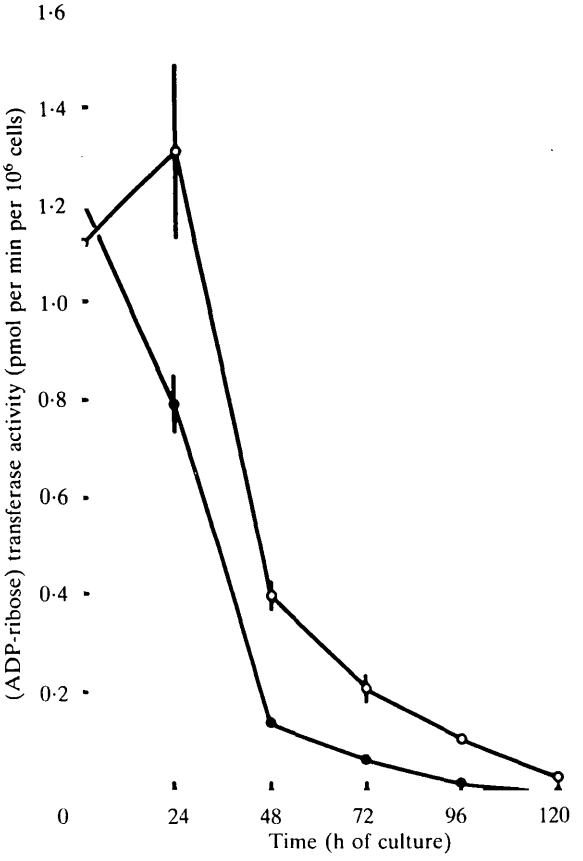

Fig. 4. (ADP-ribose) transferase activity of permeabilized cells in chick limb bud cell cultures. Four 20 μl drops of cell suspension (2×10^7/ml) were cultured. The final reaction mixture consisted of 40 mM-tris-HCl (pH = 7·8), 0·17 M-sucrose, 0·67 mM-EDTA, 13 mM-$MgCl_2$, 20 mM-2-mercaptoethanol, 1 μM [^{3}H]NAD (90 000 c.p.m./nmol) and cells. The reaction was continued for 30 min at 25 °C. The activity was obtained from the incorporation of [^{3}H]NAD into the acid-insoluble fraction, the synthesis of poly (ADP-ribose). Each point is the mean of triplicate cultures; the bars represent the standard deviation of the mean; ○, untreated cultures; ●, BAM-treated cultures.

NAD and ATP levels (Fig. 5)

Chromatographically separated components were identified on the basis of retention time and standard addition (Wehr, 1980). Elution peaks were quantitated by measuring peak height and width at half-height and determining peak area (height × width at half-height). The response factor R (mol/area) of the reference compound was determined using standard solutions containing known

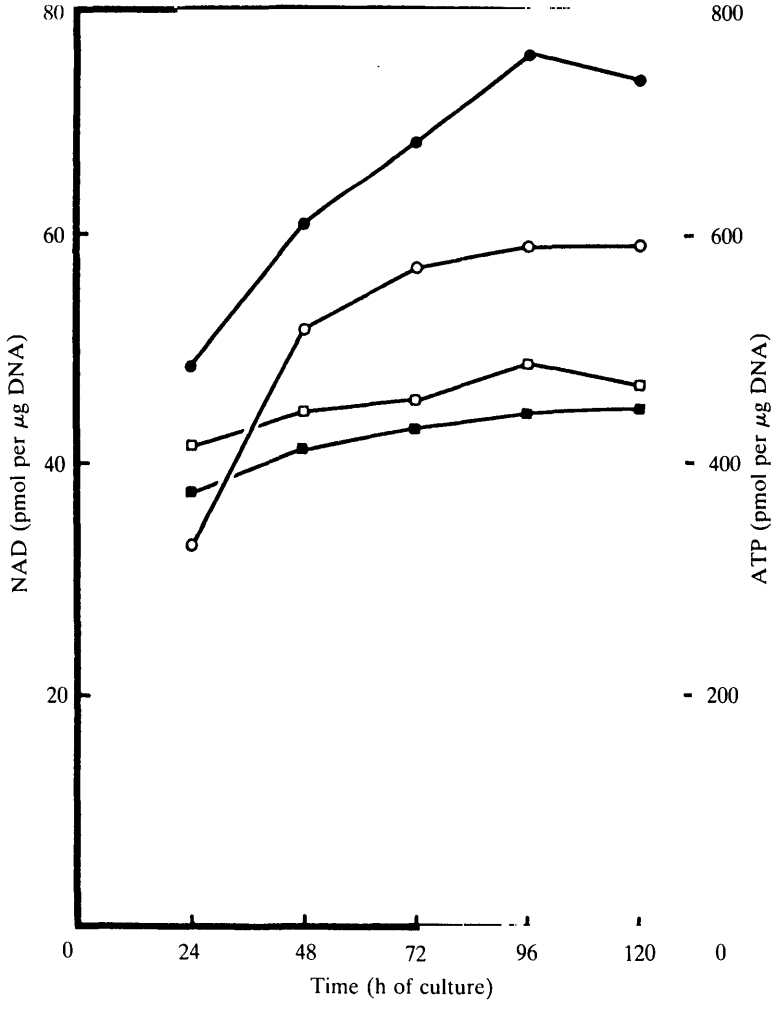

Fig. 5. The levels of NAD and ATP in chick limb bud cell cultures. A 200 μl drop of cell suspension (2×10^7/ml) was cultured. Nucleotides were extracted with 5% PCA, and PCA was neutralized with KOH. 200 μl of the supernate, obtained from triplicate cultures, were analysed by using HPLC. DNA was measured by using a modification of the method of Schmidt-Thannhauser-Schneider. Each point is the mean of two independent experiments; ○, NAD in untreated cultures; ●, NAD in BAM-treated cultures; □, ATP in untreated cultures; ■, ATP in BAM-treated cultures.

amounts of nucleotides (Zakaria & Brown, 1981). When the same batch of Partisil 10-SAX resin was used and elution conditions were kept constant, the retention times and quantitation of compounds were reproducible. Quantitation was linear in the concentration range needed for analysis of nucleotides extracted from cultured chick limb bud cells.

NAD in untreated cultures showed the lowest value at 24 h after the start of culture and increased until 96 h, but the rate of increase diminished daily. NAD in BAM-treated cultures increased similarly to the untreated but was significantly more than that in untreated cultures on each day. On the other hand, ATP did not show significant changes during the culture period, and there were no significant differences between untreated and BAM-treated cultures.

DISCUSSION

Chick limb bud mesenchymal cells differentiate into chondrocytes or non-chondrocytes, and they have been used by many as an *in vitro* model for chondrocytic differentiation; a suitable *in vitro* model for chondrocytic differentiation is the micromass culture (Ahrens *et al.* 1977). Previously, we reported the effects of inhibitors of (ADP-ribose) transferase on chondrocytic differentiation in micromass cultures; these inhibitors stimulated an early formation of cartilage nodules in micromass culture of stage 23- to 24-chick limb bud mesenchymal cells (Nishio *et al.* 1983). Others showed that these inhibitors induced differentiation of leukemic cells (Terada *et al.* 1979; Morioka *et al.* 1982*b*).

It is noteworthy to point out some observations of BAM effects on the developing chondrocytes, *in vitro*. These are schematically depicted in Fig. 6 as events *A* to *D*. Thus, in untreated cultures, *A* (decrease of (ADP-ribose) transferase activity) occurred on day 2 (Fig. 4), *B* (cell proliferation) occurred on day 3 (Fig. 1), *C* (cartilage nodule formation) occurred on day 3 (reported also in

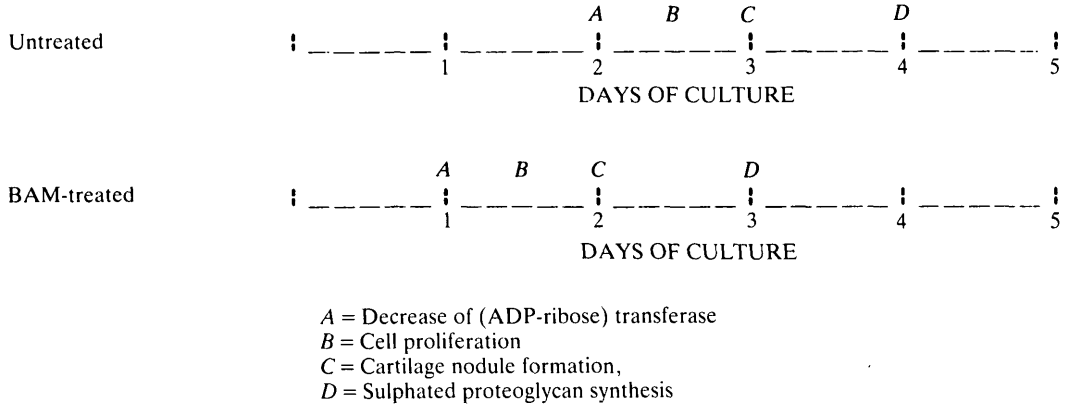

Fig. 6. Synopsis of events occurring during chick limb bud chondrogenesis, *in vitro*.

Nishio *et al.* 1983), and *D* (sulphated proteoglycan synthesis) occurred on day 4 (Fig. 2). In BAM-treated cultures, events *A* through *D* occurred one day earlier. Hence, *A* occurred on day 1, *B* occurred on day 2, *C* occurred on day 2, and *D* occurred on day 3. It is noteworthy that in BAM-treated cultures, the time interval between *A* to *D* was not shortened; all events were advanced one day earlier. In addition, colony forming assays revealed that BAM caused an eight-fold increase in chondrocytic colonies. Using a modification of a bone marrow clonal assay (Uyeki, Wierzba & Bisel, 1981), 10^5 chick limb bud cells were cloned in an agar medium with and without 3 mM-BAM. After 10 days of culture, 45 and 380 colonies were tallied for untreated and BAM-treated cultures, respectively (unpublished data). Therefore, we conclude that BAM stimulated both chondrocytic differentiation and cell proliferation.

Our studies do not agree with the earlier studies of Caplan (Caplan, Zwilling & Kaplan, 1968; Caplan, 1970; Rosenberg & Caplan, 1975) who reported that nicotinamide, an inhibitor of (ADP-ribose) transferase, inhibited chondrocytic differentiation in cultures. We suggest that this contradiction between their studies and ours may be due to several different culture conditions. Firstly, cell densities in our cultures were higher than theirs. It is well established that high cell densities, in culture, greatly augment chondrocytic differentiation (Caplan, 1970; Ahrens *et al.* 1977; Caplan, Syftestad & Osdoby, 1983; Nishio *et al.* 1983). Secondly, the differences may be due to a critical period when nicotinamide or BAM was added. In their cultures, nicotinamide was added at the end of day 2 (40–44 h after) (Caplan, 1970) or at the end of day 1 (24 h after) (Rosenberg & Caplan, 1975), while BAM was added 90 min after planting in our cultures. Although other explanations have not been excluded, BAM may preferentially affect precursor cells to a chondrocytic lineage during this period, namely, the first 24 h. We also note that BAM instead of nicotinamide was used in our study, since BAM showed higher activity for chondrocytic differentiation (Nishio *et al.* 1983).

DNA synthesis per cell did not significantly differ between untreated and BAM-treated cultures (Fig. 3). There is an apparent inconsistency between this result and the result on cell growth (Fig. 1). We suggest the possibility that the DNA assay is not sensitive enough to detect any minor differences which may occur between untreated and BAM-treated cultures. The growth curve in BAM-treated cultures is slightly steeper than that in untreated cultures after 48 h although the cell growth rate in BAM-treated cultures was significantly higher than that in untreated cultures on day 2 (between 24 h and 48 h). DNA synthesis in BAM-treated cultures on day 2 was significantly higher than that in untreated and BAM-treated cultures on day 1 although DNA synthesis in untreated cultures on day 2 did not significantly differ from that in untreated and BAM-treated cultures on day 1. It is premature to interpret the biphasic response of RNA synthesis; since RNA was not separated, *viz.*, into mRNA, tRNA, etc., further studies are required.

The nuclear enzyme, (ADP-ribose) transferase, catalyses the formation of poly (ADP-ribose)-modified chromatin proteins from NAD and is markedly dependent

on the presence of DNA containing nicks (Berger *et al.* 1978; Farzaneh *et al.* 1982). Nuclear (ADP-ribose) transferase activity is required for efficient DNA excision repair (Durkacz *et al.* 1980*a*; Durkacz, Omidiji, Gray & Shall, 1980*b*), probably because it regulates DNA ligase activity (Creissen & Shall, 1982). Many reports suggest that (ADP-ribose) transferase activity may also be involved in the control of gene expression and cell differentiation (Terada *et al.* 1979; Farzaneh *et al.* 1982; Morioka *et al.* 1982*a*,*b*; Pekala & Moss, 1983; Nishio *et al.* 1983). Since the marked decrease of (ADP-ribose) transferase activity occurred first in the sequence of temporally coupled events, we suggest that (ADP-ribose) transferase activity is involved in the control of chondrocytic differentiation.

Concerning the relationship between chondrocytic differentiation and poly (ADP-ribose), Caplan *et al.* (1979) reported that the levels of poly (ADP-ribose), *in ovo*, decreased until stage 26 in chick limb buds and increased thereafter. *In vitro*, the levels of poly (ADP-ribose) decreased before the appearance of cartilage nodules in their cultures differentiating into chondrocytes but did not show any change in their cultures differentiating into myocytes. The change of (ADP-ribose) transferase activity on cell differentiation is likely to show a different pattern for each cell type; it has been reported that the activity decreased during cell differentiation in adipocytes (Pekala & Moss, 1983), Friend leukemia cells (Morioka *et al.* 1982*a*) or HL 60 cells (Kanai *et al.* 1982). On the other hand, Farzaneh *et al.* (1982) reported that (ADP-ribose) transferase activity was elevated during myocytic differentiation.

Poly (ADP-ribose) is formed from the polymerization of NAD with the excision of nicotinamide. Therefore, we investigated the NAD levels of cultured chick limb bud cells by using a direct extraction method and HPLC (Fig. 5). NAD levels in untreated cultures markedly increased between 24 h and 48 h after the start of culture and, thereafter, gradually increased to a plateau level. This plateau curve correlated well (inversely) with diminished (ADP-ribose) transferase activity at comparable periods. A similar and more pronounced pattern was observed in BAM-treated cultures. Hence, there was an earlier and striking decrease of (ADP-ribose) transferase activity which was accompanied by higher NAD levels in BAM-treated cultures than in untreated cultures.

NAD levels, as with any biochemical constituent, represent a balance between anabolic and catabolic events. The higher NAD levels found in BAM-treated cultures could be due to an earlier decrease of (ADP-ribose) transferase activity. However, we have evidence that synthesis of NAD is also stimulated by BAM (Nakanishi, Nishio & Uyeki, 1984). Organophosphate insecticides, which induce skeletal malformations, decrease NAD levels; the administration of nicotinamide with the insecticides results in a reversal of NAD levels and skeletal malformations (Misawa, Doull, Kitos & Uyeki, 1981; Kitos *et al.* 1981). The causal relationships between NAD levels, (ADP-ribose) transferase activity and chondrocytic differentiation have not been firmly established. We suggest that higher (than untreated cultures) levels of NAD observed in BAM-treated cultures, a

consequence of the marked decrease of (ADP-ribose) transferase activity and enhanced NAD synthesis, contribute to chondrocytic differentiation.

Rosenberg & Caplan (1974) reported that NAD levels decreased between stage 22 (45 pmol/μg DNA) and 23 (30 pmol/μg DNA), and did not show any significant changes until stage 26 (35 pmol/μg DNA) when significant increases to stage 30 (50 pmol/μg DNA) were observed. In their cultures of stage 23- to 24-limb mesodermal cells, NAD levels decreased daily during chondrocytic differentiation. However, the levels in their cultured cells were 10-fold higher (350 pmol/μg DNA) than those in their *in ovo* study. On the other hand, our NAD levels in cultured cells were 33 pmol/μg DNA at 24 h after the start of culture and 57 pmol/μg DNA at 72 h, and approximated NAD levels obtained in their *in ovo* study.

ATP levels did not show any changes during chondrocytic differentiation and no differences were found between untreated and BAM-treated cultures (Fig. 5). Therefore, we suggest that ATP levels are not directly involved in chondrocytic differentiation.

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