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ENHANCED CHONDROCYTIC DIFFERENTIATION IN CHICK LIMB BUD CELL CULTURES BY INHIBITORS OF POLY(ADP-RIBOSE) SYNTHETASE

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Inhibitors of poly(ADP-ribose) synthetase, namely nicotinamide, benzamide, m-methoxybenzamide and 3-aminobenzamide, augmented chondrocytic differentiation of chick embryo limb bud mesenchymal cells, in culture. These inhibitors stimulated an early appearance and massive formation of cartilage nodules in micromass cultures of stage 23-24 chick embryos. They also induced nodule formation in micromass and cartilage colonies at micromass plating densities from stage 18-19 embryos. Benzamide, however, did not prevent differentiated chondrocytes from undergoing a pleiotypic change in cell type. These results are compatible with the putative regulatory function of poly(ADP-ribose) on cell differentiation.

Pyridine nucleotides are involved in chondrocytic differentiation during embryonic development; nicotinamide antagonists, notably 3-acetylpyridine and 6-amino nicotinamide, inhibit chondrocytic expression and produce gross bone malformation, *in vitro* and *in vivo* (1, 2). Some teratogenic consequences of organophosphate insecticides on chick embryo skeleton were correlated with decreased tissue NAD levels (3-7). Nuclear poly(adenosine diphosphate ribose) (PADPR), a major metabolite of NAD, shows an age-related fluctuation in tissue levels during chick limb bud development, *in vitro* and *in ovo* (8). Decreased cellular NAD levels were causally related to insufficient energy production which, in turn, resulted in diminished production of chondrocyte- specific macromolecules (9). Poly ADP-ribosylation of nuclear proteins, on the other hand, may be a means by which gene function is regulated by modifying higher order structures of chromatin (10, 11). Hence, participation of pyridine nucleotides in chondrocytic differentiation can be addressed at two levels of cellular organization, namely, energy metabolism and chromatin conformation. Recent advances in our knowledge of specific I-PADPRS

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(inhibitors of PADPR synthetase) may provide us with heuristic tools to probe these relationships (10-12).

Chick limb bud mesenchymal cells differentiate into chondrocytes, myocytes and fibroblasts in culture; chondrocytes are easily detectable by histochemical stains, such as Alcian blue and Toluidine blue. Using the micromass culture technique of Ahrens et al. (13), we investigated the effects of I-PADPRS on the differentiation of the mesenchymal cells into chondrocytes: A striking enhancement of chondrocytic differentiation by the inhibitors was demonstrated.

MATERIALS AND METHODS

Chemicals: Nicotinamide adenine dinucleotide (NAD), nicotinamide (NAM), benzamide (BAM), 3-aminobenzamide (3-ABAM), nicotinic acid (NAc), phorbol myristate acetate (12- O- Tetradecanoyl- phorbol- 13- acetate; TPA) and bromodeoxyuridine (BrdUrd) and Alcian blue 2G were obtained from Sigma Chemicals Co.(St. Louis, MO). Benzoic acid (BAc), 3-aminobenzoic acid (3-ABAc), m-methoxybenzamide (m-MXBAM) and m-methoxybenzoic acid (m-MXBAc) were obtained from Aldrich (Milwaukee, WI). [adenine-2,8-^{3}H]NAD (3.4 Ci/mmol) was 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 were determined following the classification of Hamburger and Hamilton (14). Limb bud cells were dissociated by enzymatic treatment for 30 min at 37°C , using a magnetic stirrer. A solution containing 0.25 percent trypsin, 0.1 percent collagenase and 30 μg DNase I were prepared in Hanks' balanced salt solution. Cells were washed once with growth medium [Ham's F12 supplemented with 10 percent fetal calf serum (KC Biological. Inc., Lenexa, KS) and penicillin/streptomycin)] and resuspended at a concentration of twenty million cells per ml (hereafter referred to as "micromass density"). Twenty μl of cell suspension were placed in the center of each well of 24 tissue culture cluster (Costar, Cambridge, M), and cells were allowed to attach to the substratum at 37° C for 90 min (13). To each culture 1 ml of growth medium was added (with or without chemicals) and cultured for the indicated periods in an atmosphere of 5 percent CO_2-95 percent air in a water-jacketed incubator at 37° C. Cultures were fed with 1 ml of fresh medium on alternate days. To obtain chondrocytes, 5 x 10^5 stage 25 limb bud cells were suspended in 10 ml growth medium and cultured over a 0.5 percent agar layer in 100 mm culture dishes for 10 days; cultures were fed every third day. Chondrocytes were dissociated to single cells with trypsin-collagenase treatment and suspended in growth medium at a concentration of 0.7 x 10^7 cells/ml. Twenty μl of cell suspension were placed in each well of a 24 well cluster plate and cultured in a manner similar to primary micro-mass cultures. After the indicated culture period cells were fixed with 95 percent ethanol and 10 percent formalin and stained with 1 percent Alcian blue at pH 1 to stain sulfated glycosaminoglycans in a cartilage matrix (15).

Poly(ADP-ribose) synthetase activity in permeabilized limb bud cells: Dissociated limb bud cells were suspended in permeabilizing buffer (which consisted of 50 mM Tris-HCl (pH=7.8), 0.25 M sucrose, 1 mM EDTA, 4 mM $MgCl_2$ and 0.05 percent Triton X-100) for 15 min in an ice bath. Cells were washed once with permeabilizing buffer and finally suspended in the buffer at a concentration of 2.5x10^7 cells/ml. The substrate mixture consisted of 100 mM Tris-HCl (pH=7.8), 30 mM $MgCl_2$ and 30 μM [^{3}H]NAD (12000 cpm/nmol). Twenty μl of cell suspension, 10 μl of substrate mixture and 3.3 μl of the chemical dissolved in water were placed in a 1.5 ml plastic test tube in an ice bath, and the reaction was continued in a 25.C water bath for 10 min.

Four-tenths ml of cold 20 percent TCA was added to the test tube to terminate the reaction, and the radioactivity in the acid-insoluble fraction was determined by liquid scintillation counting after washing the acid-precipitate with 5 ml of 5 percent TCA and 3 ml of ethanol on Whatman # 1 chromatography paper.

RESULTS

1. Inhibition of Poly(ADPR) synthetase activity: Inhibitory effects of NAM, BAM and its analogs on PADPR synthetase activity in permeabilized limb bud cells from stage 24-25 chick embryos are shown in Fig. 1. Among the chemicals, m-MXBAM showed the strongest activity. Concentrations used in the following experiments were at least ten times higher than their 100 percent inhibitory dose on PADPR synthetase activity. BrdUrd and TPA also showed inhibitory effects on PADPR synthetase.

2. I-PADPRS on cartilage nodule formation: When stage 23-24 limb bud mesenchymal cells were cultured at micromass densities, cell aggregate formation took place by the second day of culture, and these aggregates grew into Alcian blue positive-staining cartilage nodules by day 3 or 4 (13). When cultures were stained with Alcian blue at this stage, cell mass consisted of three different areas, based

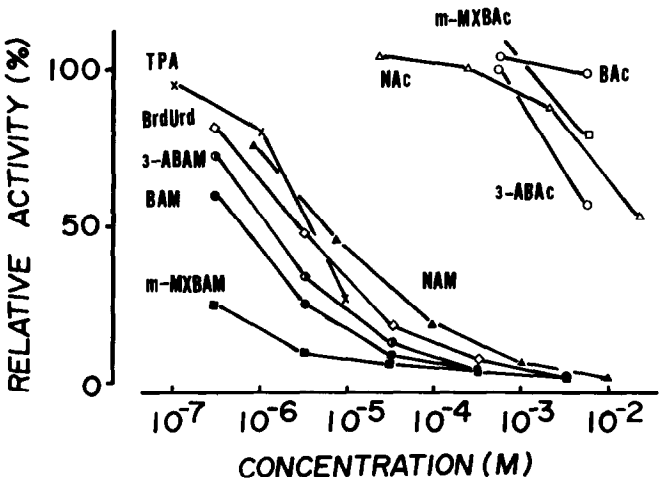

Fig. 1: Inhibition of Poly(ADPR) synthetase activity in permeabilized chick limb bud cells by NAM, BAM and their analogs. The final reaction mixture consisted of 70 mM Tris (pH=7.8), 0.17 M Sucrose, 13 mM $MgCl_2$, 0.6 mM EDTA, 10 uM [^{3}H]NAD (12000 cpm/nmol), 0.03 percent Triton X-100 and $0.5x10^6$ cells. The reaction was continued for 10 min at 25° C. The incorporation of [^{3}H]NAD into the acid-insoluble fraction without inhibitors was 11.5 pmol/min/10^6 cells. Abbreviations are: NAM= nicotinamide, BAM= benzamide, 3-ABAM= 3-aminobenzamide, m-MXBAM= m-methoxybenzamide, NAc= nicotinic acid, BAc= benzoic acid, 3-ABAc= 3-aminobenzoic acid, m-MXBAc= m-methoxybenzoic acid, BrdUrd= bromodeoxyuridine, TPA= 12- O- Tetradecanoyl- phorbol- 1,3- acetate.

Table 1
Cartilage Nodule Formation in Stage 22-24 Chick Limb Bud Cell Cultures In The Presence of Inhibitors of Poly(ADPR) Synthetase

Chemicals	Number of Nodules	Chemicals	Number of Nodules
None	96		
BAM (3 mM)	368	BAc (3 mM)	84
NAM (10 mM)	234	NAc (10 mM)	116
3-ABAM (3 mM)	193	3-ABAc (3 mM)	88
m-MXBAM (3 mM)	380	m-MXBAc (3 mM)	93
BrdUrd (30 μM)	0	BrdUrd (30 μM) + BAM (3 mM)	0
TPA (0.1 μM)	0	TPA (0.1 μM) + BAM (3 mM)	184

A 20 μl drop of cell suspension (2×10^7 cells/ml) was cultured as micromass for 5 days in the presence of the chemicals. Numbers of cartilage nodules were scored after Alcian blue staining. Mean values of triplicate cultures are shown. Abbreviations for chemicals are as in Fig. 1.

on their stainability: distinct and strongly- stained nodules, diffuse and weakly-stained central areas (background staining) and unstained peripheral areas. In the following days the nodules increased in cell numbers, fused as they increased their dimensions, and formed huge nodes in the central area of the micromass. As shown in Table 1, NAM, BAM, m-MXBAM and 3-ABAM markedly enhanced nodule formation; these I-PADPRS augmented the appearance of cell aggregates as well as the growth of nodules. Their analogs, viz., NAc, BAc, m-MXBAc and 3-ABAc, however, did not. BrdUrd, a well known inhibitor of chondrocytic differentiation (17), abolished both nodule formation and the diffuse background staining. BAM did not antagonize BrdUrd's effects. TPA also abolished nodule formation; however, a comparatively strong background staining was still evident. BAM did restore nodule formation in the presence of TPA.

3. I-PADPRS on cartilage nodule formation in cultures from various embryonic stages: The ability of limb bud cells to form cartilage nodules in culture depends on the embryonic stage; Solursh and his coworkers reported that cells from stage 17-19 did not form nodules and that stage 20 was a transitional period for chondrocytic differentiation in micromass cultures (16). We investigated the effects of NAM, BAM, 3-ABAM and m-MXBAM on the cartilage nodule formation of limb bud cells from various embryonic stages (Table 2). Nodule formation was first observed in control culture from stage 22 embryos; cells obtained before stage 22 did not aggregate in our

Table 2
Numbers of Cartilage Nodules in Limb Bud Cell Cultures From Various Embryonic Stages of Development

Chemical	Embryonic Stages			
	18-19	20-21	22-23	24-25
None	0	0	24	36
BAM (3 mM)	33	174	187	241
NAM (10 mM)	21	78	86	154
3-ABM (3 mM)	7	76	83	120
m-MXBAM (3 mM)	20	154	169	218

A 10 µl drop of cell suspension ($2x10^7$ cells/ml) was cultured as micromass for 5 days in the presence of the chemicals. Mean values of duplicate cultures are shown.

experiments. On the other hand, four I-PADPRS did induce chondrogenesis in culture from stage 18-19 embryos. It is noteworthy that BAM (3mM) produced cartilage colonies when stage 19 limb bud cells were plated at cloning densities ($5x10^3$ cells/ml).

4.Chondrogenesis of stage 23-24 micromass cultures at different plating cell densities: Cartilage nodule formation depend on plating densities (18); micromass densities (20 million cells/ml) favor nodule formation. We cultured stage 23-24 limb bud cells as micromasses at various cell densities (from 20 to 0.6 milion cells/ml). Control cultures showed nodule formation at an initial plating density of 5 million cells/ml as a micromass, but formed neither nodules nor colonies below this density. BAM induced nodule formation at a density of 2.5 million cells/ml and chondrocyte colony formation at 0.6 million cells/ml. NAM and 3-ABAM also induced chondrocytic differentiation below micromass plating densities.

Table 3
Numbers of Cartilage Nodules in Culture of Stage 23-24 Limb Bud Cells at Various Plating Cell Densities

	No. of Nodules or Colonies per Micromass Culture at Plating Cell Densities (x million cells/ml) of:					
	20	10	5	2.5	1.2	0.6
None	87	34	11	0	0	0
BAM (3 mM)	283	206	70	15	19*	20*
NAM (10 mM)	188	168	67	9*	14*	7*
3-ABAM (3 mM)	138	92	30	7*	1*	2*

Cell suspensions were prepared at the various densities, and a 20 µl drop of suspension was plated as a micromass and cultured for 5 days. Cartilage nodules and colonies were scored after Alcian blue staining. (*) indicates that only colonies were scored in these cultures. Mean values of duplicate cultures are shown.

Table 4
Cartilage Nodule Formation in Stage 23-24 Chick Limb Bud Cell Cultures Depend on the Treatment Period with BAM or NAM

Treatment Period From the Start Of Culture	Chemical		
	None	BAM (3 mM)	NAM (10 mM)
6 hr	6	6	8
12 hr	8	24	25
24 hr	12	32	25
36 hr	26	52	44
48 hr	26	146	68
72 hr	41	208	86
94 hr	37	269	125

A 20 µl drop of cell suspension ($2x10^7$ cells/ml) was cultured as a micromass. Chemicals were added at the start of the culture and removed from culture after the indicated treatment periods. Cells were washed twice with fresh medium and cultured subsequently in the absence of the chemicals. Control cultures were also washed similarly. Mean values of duplicate cultures are shown.

5. Duration of treatment with NAM and BAM on cartilage nodule formation: When stage 23-24 limb bud cells were plated at micromass density, chondrocyte specific gene amplification is said to take place during the initial 48 hr of culture (19); the presence of BrdUrd during this period abolishes the phenotypic expression of chondrocytes by interfering with gene amplification. We investigated the effects of treatment periods with NAM and BAM on chondrocytic differentiation of stage 23-24 limb bud cell cultures (Table 4). A washout during the initial 12 hour of culture was inhibitory to nodule formation and tended to obscure the results of "pulsing" chemicals during this initial period. Nonetheless, a slight, albeit significant, increase in nodule formation was brought about by an initial 12 hr-exposure of cultured cells to NAM and BAM. A 48 hr-treatment augmented nodule formation more strikingly, and further increases in the number of nodules were observed with prolonged treatments.

6. BAM on the pleiotypic change of chondrocytes to fibroblasts: While high cell densities are required for differentiation of limb bud cells to chondrocytes, low cell densities are necessary for the maintenance of the chondrocytic phenotype (18). We plated differentiated chondrocytes at micromass densities and observed their pleiotypic change to fibroblasts in the presence of BAM, BrdUrd and TPA. When these differentiated chondrocytes were cultured at micromass densities, they also formed cartilage nodules by day 2 as observed in primary limb bud cell cultures from early

Table 5
Cartilage Nodule Formation
in Secondary Chick Embryo Chondrocyte Cultures

Chemical	Number of Cartilage Nodules	
	Day 3	Day 6
None	580	658
BAM (3 mM)	375	322
BrdUrd (30 μM)	235	0
BrdUrd (30 μM) + BAM (3 mM)	248	0
TPA (0.1 μM)	0	0
TPA (0.1 μM) + BAM (3 mM)	0	0

A 20 μl drop of differentiated chondrocytes (0.7×10^7 cells/ml) was cultured as micromass for 3 and 6 days in the presence of the chemicals. Because of the larger size of the differentiated chondrocytes, 0.7×10^7 cells/ml were used as the "micromass density". Cartilage nodules were scored after Alcian blue staining.

embryos (Table 5). BAM, however, did not augment nodule formation; indeed, it inhibited the process. Although the pleiotypic change by BrdUrd did not appear until day 3, BrdUrd completely eliminated chondrocytic expression by day 6; its effect seemed to depend on cell proliferation (DNA synthesis). TPA, on the other hand, inhibited nodule formation immediately (20). BAM did not antagonize the effect of BrdUrd or TPA.

DISCUSSION

Caplan and Rosenberg (21) reported a possible regulatory role of nuclear PADPR on chondrocytic differentiation in embryonic chick limb bud mesenchymal cells; recently, they demonstrated a transient decrease in nuclear PADPR level during limb bud chondrogenesis, which occurred together with the appearance of cartilage nodules in culture (8). To gain some understanding about the loci of action of the I-PADPRS (Inhibitors of PADPR synthetase), we considered two broad possibilities. First, do the I-PADPRS act as "inducers", in the sense of erythropoietin or colony stimulating factor (26) in committing mesenchymal cells to a chondrocytic phenotype? Two, do the I-PADPRS provide a epigenetically selective environment which determines the end-stage of limb bud mesenchymal explants?

The second possibility is favored for these reasons: A prevalent view supports the notion that the limb bud mesenchymal cells are heterogeneous and contain

presumptive chondrocytes and myocytes (27-30). Moreover, these cells are thought to be in different stages of differentiation; by using quail-chick chimeras, Christ (28) showed that some limb mesenchymal cells were programmed for myocytic lineage as early as stage 14. Limb bud mesenchymal cells (before stage 20) do not form cartilage in conventional micromass cultures (16). In our studies, I-PADPRS produced chondrocytic nodules when stage 18-19 mesenchymal cells were cultured as micromasses; BAM induced cartilage colonies in stage 18-19 cells when cultured at micromass densities. Additionally, all I-PADPRS (used in this study) augmented the formation of cartilage nodules when stage 23-24 cells were cultured as micromasses at inocula below micromass densities (Table 3). Because cartilage colonies grew independent of other cells (such as fibroblasts and myoblasts), the primary effect of the I-PADPRS is due to the augmentation of chondrocytic growth and not due to permissive overgrowth, the result of inhibition of other cell lines. Taken together, the collective data suggest that the differentiating processes are pre-programmed in these early mesenchymal cells and that environmental factors (including I-PADPRS) favor the further differentiation to a chondrocytic lineage. Therefore, we consider the second possibility as the more likely one.

Augmentation of chondrocytic differentiation by I-PADPRS depended on the developmental stage of the embryo, in ovo (Table 2), and the treatment period with I-PADPRS (Table 4, overall period of 5 days); these studies suggest a post-commitment effect of the I-PADPRS. Moreover, the augmentation of chondrocytic differentiation by the I-PADPRS was specific; indeed, BAM inhibited the maintenance of a previously established chondrocytic phenotype (Table 5).

Bromodeoxyuridine and TPA are inhibitors of chondrocytic differentiation. The use of these two agents permitted us to further dissect the role of BAM on the differentiating process (Table 1 and 5). The inhibitory effect of BrdUrd is due to its incorporation into DNA (Strom and Dorfman, 19); BAM did not antagonize this effect of BrdUrd. TPA exerted different effects on mesenchymal cells and on differentiated chondrocytes. Whereas TPA prevented nodule formation in mesenchymal cells, Alcian blue staining (diffuse background staining) was not prevented. Addition of BAM restored nodule formation in these differentiating mesenchymal cells.

On the other hand, TPA completely inhibited chondrocytic expression of differentiated chondrocytes; the prevalent cells in these cultures were "fibroblastic" (18). The addition of BAM to these cultures did not restore these "fibroblasts" to a chondrocytic phenotype. Therefore, BAM was able to restore nodule formation in differentiating, mesenchymal cells and unable to alter the TPA- altered phenotype (viz., fibroblasts) of older, committed cells.

Although alternate explanations have not been excluded, an explanation which appeals to us is the following: The primary locus of action of TPA is the cell membrane (22) and subsequent regulatory events turnoff the chondrocytic phenotype. One such regulatory mechanism is poly-ADP ribosylation of chromatin proteins in differentiating chondrocytes. BAM, an inhibitor of PADPR synthetase, can prevent poly-ADP ribosylation of chromatin proteins. Hence, BAM influences chondrocytic differentiation at the epigenetic level (TPA), but does not influence differentiation at the genetic level (BrdUrd). Induction of differentiation by I-PADPRS was reported in mouse Friend erythroleukemia cells (23-24). Also, transient decreases in nuclear PADPR levels occurring during the differentiation of human HL-60 leukemic promyelocytes were reported recently (25). Collectively, these reports and our present study support the notion that poly ADP-ribosylation is a regulatory mechanism in cellular differentiation.

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