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Prostaglandin synthesis during the course of limb cartilage differentiation *in vitro*

STEVEN W. GAY AND ROBERT A. KOSHER

Department of Anatomy, University of Connecticut Health Center, Farmington, CT 06032, U.S.A.

SUMMARY

In the present study we have used radiometric thin layer chromatography (TLC) and radioimmunoassay (RIA) to examine the synthesis of various prostaglandins (PGs) during the progressive chondrogenic differentiation limb mesenchymal cells undergo in micromass culture. Throughout the 3-day culture period, [^{3}H]arachidonic acid (AA) is metabolized to compounds which comigrate with authentic PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, TxB_2, and PGD_2. In micromass cultures prepared from the cells of whole stage-23/24 wing buds, all ^{3}H-AA metabolites are produced in relatively small amounts during the initial period of culture, i.e. prior to the formation of extensive prechondrogenic cellular aggregates. Concomitant with maximum aggregate formation and the initiation of cartilage differentiation, there is a striking and progressive increase in the production of all the major classes of PGs from ^{3}H-AA. PG production from ^{3}H-AA is also at a maximum during the onset of chondrogenesis in micromass cultures prepared from the distal subridge mesenchymal cells of stage-25 wing buds in which more rapid, extensive, and homogeneous cartilage differentiation occurs. To complement these TLC studies, RIA has been used to examine the amount of various PGs synthesized from endogenous substrates by micromass culture homogenates at various times during *in vitro* chondrogenesis. These RIA studies also indicate that PG production is highest during periods of culture which coincide with the onset of overt chondrogenesis in both stage-23/24 whole limb and stage-25 subridge mesoderm micromass cultures. RIA indicates that PGE_2 is the predominant PG produced from endogenous substrates during 1 h incubations at the onset of chondrogenesis, while radiometric TLC indicates compounds which comigrate with $PGF_{2\alpha}$ are the major class of ^{3}H-AA metabolites which accumulate during that time. This qualitative difference very likely reflects metabolism of parent PG compounds during the long (12 h) labelling and postlabelling incubations utilized in the TLC analyses. The temporal correlation between PG production and the initiation of chondrogenesis *in vitro* is consistent with previous studies implicating PGs in the regulation of limb cartilage differentiation.

INTRODUCTION

A crucial event in limb cartilage differentiation *in vivo* and *in vitro* is a transient cellular condensation or aggregation process in which prechondrogenic mesenchymal cells become closely juxtaposed to one another prior to depositing a cartilage matrix. During this process a cell–cell interaction, cell shape change, or some other event occurs which is necessary to trigger the chondrogenic differentiation of the cells (see Kosher, 1983*a*,*b*; Solursh, 1983 for reviews). The

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critical condensation process may be initiated, at least in part, by a progressive decrease in the accumulation of extracellular hyaluronate (Kosher, Savage & Walker, 1981; Toole, 1973). The nature of the cell–cell interaction occurring during condensation is not well understood, although it has been suggested that fibronectin or type I collagen, which are present in relatively high amounts along the surfaces of the closely apposed cells, may be involved (Dessau, von der Mark, von der Mark & Fischer, 1980; Silver, Foidart & Pratt, 1981; Kosher, Walker & Ledger, 1982; Tomasek, Mazurkiewicz & Newman, 1982). Recent studies suggest that a change in the shape of the cells from a flattened mesenchymal morphology to a rounded configuration may play an important rôle in the process (Archer, Rooney & Wolpert, 1982; Zanetti & Solursh, 1984).

Cyclic AMP appears to have a crucial part in the regulation of limb cartilage differentiation (see Kosher, 1983*b*; Elmer, Smith & Ede, 1981 for reviews). For example, agents that elevate cAMP levels promote limb chondrogenesis in organ culture (Kosher, Savage & Chan, 1979*b*; Kosher & Savage, 1980) and cell culture (Ahrens, Solursh & Reiter, 1977; Solursh, Reiter, Ahrens & Vertel, 1981). Furthermore, an increase in cAMP content correlating with the onset of chondrogenesis in high-density cell culture has been reported (Solursh, Reiter, Ahrens & Pratt, 1979; Ho, Greene, Shanfeld & Davidovitch, 1982; Biddulph, Sawyer & Smales, 1984; see also Elmer *et al.* 1981). It has been suggested that an elevation of cellular cAMP levels occurring during the condensation phase of chondrogenesis may trigger the differentiation of the cells (Kosher & Savage, 1980; Solursh *et al.* 1981; Elmer *et al.* 1981).

Several recent studies have indirectly implicated certain prostaglandins (PGs), in particular PGE_2, in the regulation of limb cartilage differentiation. Prostaglandins are critically important *local* regulators of a variety of cellular processes in a number of biological systems (Samuelsson *et al.* 1978; Kuehl & Egan, 1980), and their regulatory effects in many systems are mediated by cAMP (Hammarstrom, 1982; Kuehl, 1974; Martin & Partridge, 1980). Exogenous PGE_2 stimulates limb chondrogenesis in both organ culture (Kosher & Walker, 1983) and high density cell culture (Gay & Kosher, 1984; Chepenik, Ho, Waite & Parker, 1984). In each of these systems the stimulatory effect of PGE_2 is potentiated by phosphodiesterase inhibitors, suggesting its regulatory effect on chondrogenesis is mediated by cAMP (Kosher & Walker, 1983; Gay & Kosher, 1984). In addition, PGE_2 elicits a striking increase in the cAMP content of limb mesenchymal cells, indicating these cells do indeed possess adenylate cyclase-coupled receptors for this molecule (Parker, Biddulph & Ballard, 1981; Ballard & Biddulph, 1983; Kosher & Gay, 1985). Furthermore, the relative effectiveness of various PGs in stimulating cAMP accumulation in limb mesenchymal cells directly reflects the relative potencies of these same molecules in stimulating *in vitro* chondrogenesis (Kosher & Gay, 1985). On the basis of these observations, we have suggested that prostaglandins produced during the condensation phase of chondrogenesis might be involved in regulating limb cartilage differentiation by acting as local modulators of cAMP formation.

In view of the studies implicating prostaglandins in the regulation of chondrogenesis, it was of paramount importance to demonstrate directly the production of prostaglandins during the course of limb cartilage differentiation, and to determine if qualitative or quantitative changes in the pattern of prostaglandin production occur during different phases of the process. Accordingly, in the present study we have utilized radiometric thin layer chromatography and radioimmunoassay to examine the synthesis of various prostaglandins during the progressive chondrogenic differentiation limb mesenchymal cells undergo in micromass culture. Our results demonstrate that various prostaglandins including PGE_2, $PGF_{2\alpha}$, prostacyclin (PGI_2), thromboxane A_2 (TxA_2), and PGD_2 are indeed produced by limb mesenchymal cells undergoing chondrogenesis *in vitro*. Furthermore, the results indicate that prostaglandin production is at a maximum during periods of culture coinciding with the onset of chondrogenesis as characterized by the formation of prechondrogenic aggregates and their initial conversion into cartilage nodules. These observations provide support for the suggestion that endogenous prostaglandins may be involved in regulating limb cartilage differentiation.

MATERIALS AND METHODS

Preparation of cultures

Wing buds were removed from stage-23, -24, and -25 (Hamburger & Hamilton, 1951) embryos of White Leghorn chicks and placed into Hanks' balanced salt solution (HBSS) or Puck's saline G. Distal wing bud tips (subridge regions) were cut away from the stage-25 limb buds as previously described (Kosher, Savage & Chan, 1979*a*). The size of the excised subridge regions was approximately 0·3–0·4 mm from the distal apex of the tissue to the proximal cut edge (see Kosher *et al.* 1979*a* for a photograph). Ectoderm was removed from the whole stage-23 and -24 limb buds and the subridge regions of stage-25 limb buds following brief treatment with trypsin as previously described (Kosher *et al.* 1979*a*).

Micromass cultures were then prepared from the cells of whole stage-23 to -24 wing buds or the subridge mesoderm of stage-25 wing buds as previously described (Ahrens *et al.* 1977; Gay & Kosher, 1984). Briefly, the tissue was washed with calcium- and magnesium-free HBSS (CMF-HBSS), and incubated for 20 min at 37 °C in 0·25 % trypsin in CMF-HBSS. The tissue was then washed with F12 medium containing 10 % foetal calf serum (FCS) and antibiotics, and the cells were mechanically dissociated by repeated pipetting. The concentration of the resulting suspensions was determined with a haemacytometer, and the cell concentration adjusted to 2×10^7 cells ml^{-1}. Micromass cultures were then prepared by dispensing 10 μl drops of the cell suspensions (i.e. 2×10^5 cells) onto 35-mm Falcon tissue culture dishes. The cultures were incubated for 1·5–2 h at 37 °C in a humidified CO_2 incubator to allow for cell attachment, after which the cultures were supplied with 2·0 ml of medium (F12 supplemented with 10 % FCS and antibiotics).

Radiometric thin layer chromatographic analysis of prostanoid production

At zero time, 12 h, 24 h, 36 h, and 48 h following the initiation of culture, micromass cultures were incubated for 12 h in 1·0 ml of medium (F12 containing 10 % FCS) supplemented with 6·0 μCi of [5,6,8,9,11,12,14,15-^{3}H]arachidonic acid (100–131 Ci $mmol^{-1}$; Amersham). After each 12 h labelling period the medium was removed, and the radioactivity in an aliquot of it determined to estimate the uptake of [^{3}H]arachidonic acid by the cells. The cells were washed three times with serum-free F12 medium, fresh non-radioactive medium (F12 with 10 % FCS) was added, and the cultures incubated for an additional 12 h. The culture medium was then

quick frozen and stored at −20 °C prior to analysis. The cells were scraped into a precise quantity of HBSS, sonicated, and an aliquot of the sonicate removed for DNA determination by the fluorometric procedure of Brunk, Jones & James (1979) using chicken blood DNA as standard. In each experiment at each time point, blank incubations were performed precisely as described above in the absence of cells in order to control for the possible presence of prostanoid-like contaminants in the isotope and the possibility of spontaneous conversion of the labelled arachidonic acid into prostanoid-like molecules during the 12 h labelling and 12 h postlabelling incubation periods. When the medium of such blank incubations was analysed by thin layer chromatography as described below, small, but detectable, amounts of radioactivity above background were found comigrating with several authentic prostaglandin standards. By comparing the distribution of radioactivity in chromatographs of these procedure blanks with the distribution in experimental samples, the amount of radioactivity associated with each prostaglandin that was unequivocally due to [^{3}H]arachidonic acid metabolism by the cells at each time point could be determined.

Labelled prostanoids were extracted from the culture medium using the octadecylsilyl (ODS) silica column procedure of Powell (1980, 1982). The medium was acidified to pH 3·25 with 1·0 M-citric acid, and loaded onto SEP-PAK C_{18} cartridges (comprising ODS silica; Waters Associates) that had been prewashed with absolute ethanol followed by distilled water as described (Powell, 1980, 1982). The SEP-PAK C_{18} cartridges were then washed successively with 15 % ethanol and petroleum ether, after which prostanoids were eluted from the columns with ethyl acetate or methyl formate (Powell, 1980, 1982). The methyl formate or ethyl acetate fraction was evaporated under a stream of nitrogen. The dried residue was dissolved in chloroform/methanol (2:1, V/V), and applied to thin layer chromatographic plates along with the authentic prostaglandin standards PGE_2, $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$, thromboxane B_2, PGD_2, PGA_2, and arachidonic acid (Upjohn and Sigma). For development in solvents A or B (see below) the samples were applied to Brinkmann silica gel G-25 precoated plastic TLC sheets, and for development in solvent C (see below) the samples were applied to the preadsorbent zone of Whatman LKD silica gel precoated TLC plates as recommended by Salmon & Flower (1982). The chromatographs were equilibrated and developed twice in one of the three following solvent systems: ethyl acetate/acetic acid (97/3, V/V, solvent A; see Malemud, Moskowitz & Hassid, 1981); ethyl acetate/acetic acid/water (94/6/0·7, V/V/V, solvent B); or, the organic phase of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (110/50/20/100, V/V/V/V, solvent C; see Salmon & Flower, 1982). After development, prostaglandin standards were visualized following brief exposure of the chromatograms to iodine vapour. Appropriate areas were cut out (when plastic TLC sheets were utilized) or scraped into scintillation vials, supplied with 10 ml of Aquasol-2 (New England Nuclear), and radioactivity determined with a Beckman LS-6800 scintillation counter equipped with automatic quench correction.

Radioimmunoassay of prostanoid production

The production of various prostaglandins from endogenous substrates by micromass culture homogenates during different phases of cartilage differentiation was determined by radioimmunoassay (RIA) as follows. At various times following the initiation of culture, cells were washed and harvested in Puck's saline G, and sonicated on ice. An aliquot of the sonicate was removed for DNA determination (Brunk *et al.* 1979), and the remainder incubated for 60 min at 37 °C in a shaking water bath. In some experiments incubations were performed in the presence of indomethacin by adding small aliquots of a concentrated solution of the drug in absolute ethanol. In these experiments equivalent amounts of ethanol were added to control samples. Following the 60 min incubation period, the samples were centrifuged at 2700 r.p.m. for 30 min at 4 °C, and aliquots of the supernatants utilized for RIA. In all experiments, blank incubation mixtures lacking cells were set up and carried through the entire procedure to determine if the incubation conditions, medium, or other factors had any non-specific effect on the RIAs. No prostaglandins of any type assayed were detectable in such procedure blanks, indicating a lack of any non-specific interference of the procedure with the assays.

The concentrations of PGE_2, 6-keto $PGF_{1\alpha}$ (the stable conversion product of prostacyclin, PGI_2), and thromboxane B_2 (the stable product of thromboxane A_2) were determined using (^{125}I) RIA kits purchased from New England Nuclear, and $PGF_{2\alpha}$ content was determined using

a (^{3}H) RIA kit purchased from Seragen, Inc. The cross reactivity of the antibodies supplied with these kits are as follows: Anti-PGE_2 cross reacts by 3·7 % with PGE_1 and by less than 0·5 % with other prostaglandins; anti-6-keto $PGF_{1\alpha}$ cross reacts by 2·6 % with $PGF_{2\alpha}$ and by less than 2 % with other prostaglandins; anti-TXB_2 cross reacts by 3·9 % with PGD_2 and by less than 0·3 % with other prostaglandins; and, anti-$PGF_{2\alpha}$ cross reacts by less than 2 % with all prostaglandins except $PGF_{1\alpha}$ with which it does cross react. RIAs were performed precisely according to the instructions supplied by the manufacturers of the RIA kits. Briefly, the samples and appropriate known standards were incubated overnight at 4°C in the presence of the appropriate antibody and ^{125}I-labelled or ^{3}H-labelled tracer. In the PGE_2, 6-keto $PGF_{1\alpha}$, and TxB_2 RIAs, antigen–antibody complexes were separated from free antigen by precipitation with polyethylene glycol in the presence of carrier immunoglobulin, and the radioactivity in the precipitate determined with a gamma counter. In the $PGF_{2\alpha}$ RIA, unbound antigen was precipitated by the addition of dextran-coated charcoal, the supernatants containing the antigen–antibody complexes were added to Aquasol-2, and radioactivity determined by liquid scintillation counting.

RESULTS

In our initial experiments, radiometric thin layer chromatography (TLC) was used to examine the metabolism of [^{3}H]arachidonic acid (^{3}H-AA) into prostaglandins (PGs) at various times during the micromass culture of cells of whole stage-23/24 limb buds. Chondrogenesis in such cultures proceeds as previously described in detail (see, for example, Ahrens *et al.* 1977; Lewis, Pratt, Pennypacker & Hassell, 1978; Gay & Kosher, 1984). Briefly, by the end of the first day of culture, prechondrogenic aggregates of cells begin to be detected in various discrete regions of the culture. Between 24 and 48 h, there is a progressive increase in the size and number of these prechondrogenic aggregates. At 48 h some of the aggregates begin to be converted into cartilage nodules which stain faintly with Alcian blue (pH 1·0). By the end of day 3, virtually all of the aggregates have been converted into cartilage nodules that stain intensely with Alcian blue.

A qualitative analysis of the relative amounts of the major classes of PGs detected by radiometric TLC during the micromass culture of whole stage-23/24 limbs is shown in Table 1. During consecutive 12 h labelling and postlabelling incubation periods throughout the first three days of culture, ^{3}H-AA is metabolized to compounds which comigrate with the authentic PG standards, PGE_2, $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$ (which is the stable metabolite of the extremely labile PG, prostacyclin), TxB_2 (the stable metabolite of labile TxA_2) and PGD_2. Significantly, essentially the same qualitative pattern of PG production from ^{3}H-AA is observed in three distinct solvent systems (Table 1). One of the noteworthy features of this analysis is that ^{3}H-AA metabolites which comigrate with $PGF_{2\alpha}$ constitute a relatively minor class of PGs during the initial period of culture, i.e., prior to extensive aggregate formation, and became the predominant metabolites detectable during the maximum formation of prechondrogenic aggregates and their conversion into cartilage nodules (Table 1).

It is important to emphasize that when the cells of whole stage-23/24 limb buds are subjected to micromass culture, the cartilage nodules which differentiate are separated from one another by a considerable quantity of non-chondrogenic fibroblastic-like tissue (see for example, Ahrens, Solursh, Reiter & Singley, 1979; Gay & Kosher, 1984). Furthermore, numerous myogenic cells differentiate

throughout the culture (Ahrens *et al.* 1979; Gay & Kosher, 1984). In view of the heterogeneity of the cell types that differentiate in stage-23/24 whole limb cultures, it was critical to also examine PG production in micromass cultures

Table 1. *Relative amounts of various [^{3}H]arachidonic-acid-labelled metabolites detected at various times during the micromass culture of cells of whole stage-23/24 wing buds as determined by radiometric thin layer chromatography in three different solvent systems*

		Solvent†			
Culture period*	Prostanoid	A(%)‡	B(%)	C(%)§	AV (%+S.E.M.)$^+$
1	6-keto-$PGF_{1\alpha}$	{30·5	32·5	24·2	26·0 ± 4·1
	$PGF_{2\alpha}$		11·5	12·8	11·2 ± 1·3
	PGE_2	28·4	23·4	{37·2	25·7 ± 1·8
	TxB_2	17·7	7·7		12·4 ± 3·6
	PGD_2	23·4	25·0	25·8	24·7 ± 0·9
2	6-keto-$PGF_{1\alpha}$	{31·3	34·2	26·5	27·3 ± 4·7
	$PGF_{2\alpha}$		10·1	20·1	13·5 ± 4·0
	PGE_2	28·0	24·6	{31·6	24·1 ± 2·9
	TxB_2	17·5	14·0		14·4 ± 2·0
	PGD_2	23·2	17·2	21·8	20·7 ± 2·2
3	6-keto-$PGF_{1\alpha}$	{34·2	20·4	16·9	16·3 ± 3·1
	$PGF_{2\alpha}$		30·2	44·7	32·5 ± 7·9
	PGE_2	22·7	18·7	{23·2	18·2 ± 3·4
	TxB_2	16·6	14·8		13·8 ± 2·4
	PGD_2	26·5	15·9	15·1	19·2 ± 4·5
4	6-keto-$PGF_{1\alpha}$	{63·5	21·9	14·9	18·7 ± 2·5
	$PGF_{2\alpha}$		37·5	48·9	43·6 ± 4·1
	PGE_2	14·7	19·9	{23·0	16·9 ± 1·9
	TxB_2	6·2	8·6		7·2 ± 0·9
	PGD_2	15·6	12·1	13·2	13·6 ± 1·2
5	6-keto-$PGF_{1\alpha}$	{54·3	20·8	11·6	16·2 ± 3·3
	$PGF_{2\alpha}$		33·9	43·0	38·4 ± 3·2
	PGE_2	13·0	15·7	{24·3	15·3 ± 1·5
	TxB_2	4·7	7·2		6·3 ± 1·0
	PGD_2	28·0	22·4	21·1	23·8 ± 2·6

* At 12 h intervals throughout the initial 3 days of culture, cells were prelabelled for 12 h with [^{3}H]arachidonic acid, and then incubated for an additional 12 h to allow labelled metabolites to be released into the medium. Culture period 1 represents prelabelling with [^{3}H]arachidonic acid from 0–12 h followed by a 'chase' from 12–24 h; culture period 2, prelabelling from 12–24 h, chase from 24–36 h; culture period 3, 24–36 h prelabelling, 36–48 h chase; culture period 4, 36–48 h prelabelling, 48–60 h chase; culture period 5, 48–60 h prelabelling, 60–72 h chase.

† See Materials and Methods for detailed descriptions of solvent systems A, B, and C.

‡ In solvent system A, 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ co-migrate.

§ In solvent system C, PGE_2 and TxB_2 co-migrate.

$^+$ In calculating the average, the relative amounts of 6-keto-$PGF_{1\alpha}$ and $PGF_{2\alpha}$ in solvent A in which they co-migrate was assumed to be equal to the average relative amounts of these two prostanoids in solvents B and C in which they do not comigrate. Similarly, the relative amounts of PGE_2 and TxB_2 in solvent C in which they comigrate was assumed to be equal to their average relative amounts in solvent A and B in which they do not co-migrate.

prepared from the mesoderm directly subjacent to the AER of stage-25 wing buds. Stage-25 subridge mesoderm consists of a virtually homogeneous population of undifferentiated chondrogenic progenitor cells, which uniformly progress through the phases of chondrogenesis in micromass culture and form a virtually uniform sheet of cartilage with little, if any, non-chondrogenic tissue detectable (see Gay & Kosher, 1984). In such cultures, widespread and extensive prechondrogenic aggregations of cells are detected by 16–24 h, and by 36–48 h an extensive and virtually uniform Alcian-blue-staining cartilage matrix has formed throughout the cultures. As shown in Table 2, in stage-25 subridge micromass cultures, as was the case in whole stage-23/24 cultures, ^{3}H-AA-labelled metabolites which comigrate with PGE_2, $PGF_{2\alpha}$, 6-keto $PGF_{1\alpha}$, TxB_2, and PGD_2 are produced throughout the culture period. In the stage-25 subridge cultures, in which cartilage differentiation proceeds more rapidly than in stage-23/24 whole limb cultures, ^{3}H-AA-labelled metabolites that comigrate with $PGF_{2\alpha}$ are the predominant class of PGs produced during the first day or two of culture, at which time widespread aggregations of cells are forming and initiating chondrogenesis. Thus compounds which comigrate with $PGF_{2\alpha}$ are the predominant ^{3}H-AA metabolites detected by radiometric TLC at the onset of chondrogenesis in both culture systems.

Quantitative analyses of changes in the amounts (d.p.m. μg^{-1} DNA) of the various ^{3}H-AA metabolites detected by TLC during the course of chondrogenic differentiation in stage-23/24 whole limb and stage-25 subridge mesoderm micromass cultures are shown in Figs 1 and 2, respectively. In stage-23/24 whole limb cultures, all ^{3}H-AA-labelled PG metabolites are present in relatively small amounts during the initial period of culture, i.e. prior to extensive aggregate formation (Fig. 1). Beginning at about 36–48 h, which corresponds to maximum

Table 2. *Relative amounts of various [^{3}H]arachidonic-acid-labelled metabolites detected at various times during the micromass culture of cells from the distal subridge mesoderm of stage-25 wing buds as determined by radiometric thin layer chromatography*

	Culture period*†				
Prostanoid	1(%)	2(%)	3(%)	4(%)	5(%)
6-keto-$PGF_{1\alpha}$	19·6 ± 2·9	18·7 ± 2·0	22·9 ± 4·0	32·1 ± 6·5	22·8 ± 5·3
$PGF_{2\alpha}$	33·4 ± 5·6	39·6 ± 6·2	31·7 ± 4·1	23·5 ± 3·9	28·6 ± 7·9
PGE_2	21·9 ± 2·7	19·4 ± 4·0	23·3 ± 4·6	19·5 ± 3·5	17·8 ± 4·0
TxB_2	9·2 ± 1·8	11·1 ± 2·3	9·9 ± 3·6	15·5 ± 2·9	15·4 ± 2·1
PGD_2	15·9 ± 3·7	11·2 ± 2·8	12·2 ± 1·7	9·4 ± 3·4	15·4 ± 3·2

* At 12 h intervals throughout the initial 3 days of culture, cells were prelabelled for 12 h with [^{3}H]arachidonic acid, and then incubated for an additional 12 h to allow labelled metabolites to be released into the medium. Culture period 1 represents prelabelling with [^{3}H]arachidonic acid from 0–12 h followed by a chase from 12–24 h; culture period 2, 12–24 h prelabelling, 24–36 h chase; culture period 3, 24–36 h prelabelling, 36–48 h chase; culture period 4, 36–48 h prelabelling, 48–60 h chase; culture period 5, 48–60 h prelabelling, 60–72 h chase.

† Values are the means of six determinations ± S.E.M.

aggregate formation and the initiation of cartilage differentiation, there is a striking increase in the accumulation of all the major classes of PGs from ^{3}H-AA (Fig. 1). During that period there is about a 20-fold increase in the accumulation of ^{3}H-AA metabolites that co-migrate with $PGF_{2\alpha}$; a greater than three-fold increase in metabolites that comigrate with PGE_2, 6-keto-$PGF_{1\alpha}$, and TxB_2; and, a two-fold increase in metabolites that comigrate with PGD_2 (Fig. 1). During subsequent periods of culture production of all ^{3}H-AA metabolites continues to increase (Fig. 1). In stage-25 subridge mesoderm micromass cultures, in which cartilage differentiation occurs more rapidly, extensively, and homogeneously, the accumulation of all classes of ^{3}H-AA-labelled PGs is highest during the first two days of culture (Fig. 2), which is the period that corresponds to the formation of widespread prechondrogenic aggregations of cells and their initial differentiation into cartilage. Following overt cartilage differentiation in the stage-25 subridge cultures, the production of all classes of PGs from ^{3}H-AA sharply declines (Fig. 2). Thus in both stage-23/24 whole limb and stage-25 subridge cultures, the production of all classes of PGs from ^{3}H-AA is high during periods which coincide with the onset of chondrogenesis.

Our radiometric TLC analysis of PG production during chondrogenesis should be considered tentative and interpreted cautiously for several reasons. First of all, in order to ensure adequate incorporation of ^{3}H-AA into PGs, we utilized rather long (12 h) labelling and postlabelling incubation periods. In this regard it should be pointed out that virtually all primary PGs that are produced by a variety of cells undergo fairly rapid metabolism and interconversions into compounds which do not necessarily comigrate with the parent PG that was initially produced (Samuelsson *et al.* 1978; Hall & Behrman, 1982; Granstrom & Kindahl, 1983). In fact, a major metabolite of PGE_2 is $PGF_{2\alpha}$ or compounds which comigrate with $PGF_{2\alpha}$ on TLC (Hall & Behrman, 1982; Granstrom & Kindahl, 1983). In addition, we would like to point out that in two of the eight experiments in which we used radiometric TLC to monitor PG production during the micromass culture of whole stage-23/24 limb buds (Table 1 and Fig. 1), large increases in ^{3}H-AA metabolism during later periods of culture were not observed. Therefore, to circumvent some of the inherent problems associated with our TLC analyses, we used radioimmunoassay (RIA) to examine the synthesis of PGE_2, $PGF_{2\alpha}$, prostacyclin, and TxA_2 from endogenous substrates by micromass culture homogenates during different phases of cartilage differentiation (see Materials and Methods).

Fig. 3 demonstrates the amount (pg μg^{-1} DNA) of the various PGs synthesized from endogenous substrates during 1 h incubations by stage-23/24 whole limb micromass culture homogenates at various times following the initiation of culture. All the PGs assayed are produced at relatively low levels by homogenates of 12 h micromass culture (i.e. prior to aggregate formation). From 12 to 48 h, which is the period of culture during which prechondrogenic aggregates are forming and undergoing their initial differentiation into cartilage nodules, there is a striking and progressive increase in PG production. PGE_2 synthesis by micromass culture homogenates is three-fold, seven-fold, and eleven-fold greater at

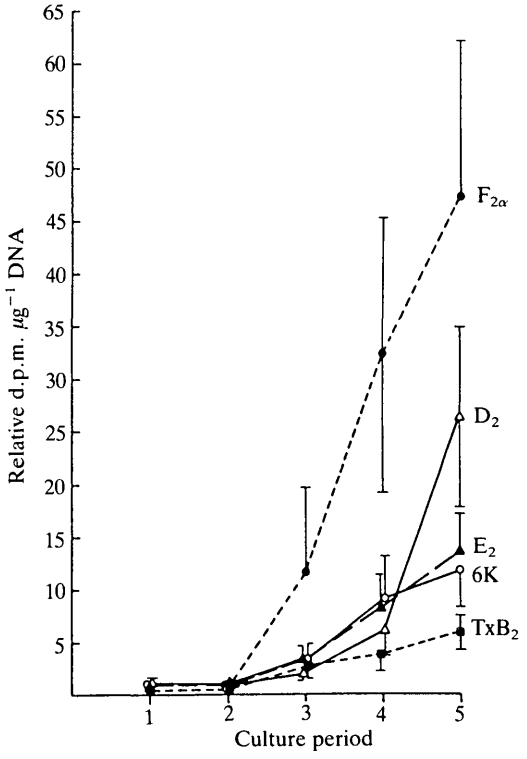

Fig. 1. Amount of various [^{3}H]arachidonic-acid-labelled metabolites produced at various times during the micromass culture of cells of whole stage-23/24 wing buds as determined by radiometric thin layer chromatography. At 12 h intervals throughout the 3 days of culture, cells were prelabelled for 12 h with [^{3}H]arachidonic acid, and then incubated for an additional 12 h to allow labelled metabolites to be released into the medium. Culture period 1 in the graph represents prelabelling with [^{3}H]arachidonic acid from 0–12 h followed by a 'chase' from 12–24 h; culture period 2 represents prelabelling from 12–24 h followed by a chase from 24–36 h; culture period 3 represents prelabelling from 24–36 h followed by a chase from 36–48 h; culture period 4, 36–48 h prelabelling, 48–60 h chase; culture period 5, 48–60 h prelabelling, 60–72 h chase. In order to validly compare data obtained from eight separate experiments performed over the course of two years, the data from each experiment were normalized as follows: The amount (d.p.m. μg^{-1} DNA) of each of the [^{3}H]arachidonic-acid-labelled metabolites produced during each period of culture was divided by the amount (d.p.m. μg^{-1} DNA) of PGE_2 produced during culture period 1 in that experiment (= relative d.p.m. μg^{-1} DNA). Each value thus represents the mean of 8 determinations ± S.E.M. $PGF_{2\alpha}$ ($F_{2\alpha}$, ●----●); PGE_2 (E_2, ▲---▲); PGD_2 (D_2, △——△); 6-keto-$PGF_{1\alpha}$ (6K, ○——○); TxB_2 (■----■).

24 h, 36 h, and 48 h respectively than it is at 12 h (Fig. 3). During this same period the production of $PGF_{2\alpha}$ and TxA_2 (as assayed by the amount of its stable metabolite, TxB_2) increases two-fold (Fig. 3). In contrast, prostacyclin (as assayed by the amount of its stable metabolite, 6-keto-$PGF_{1\alpha}$) is produced at low levels during all periods of culture (Fig. 3). PGE_2 is by far the predominant PG produced during 1 h incubations by micromass culture homogenates throughout the onset of

chondrogenesis. Homogenates of 48 h micromass cultures produce three-fold more PGE_2 than $PGF_{2\alpha}$, five-fold more PGE_2 than TxA_2, and twenty-fold more PGE_2 than prostacyclin (Fig. 3). Following the initiation of chondrogenesis (i.e. at 60 h), the production of all PGs by micromass culture homogenates sharply decreases (Fig. 3). Interestingly, PG production again increases at 72 h of culture (Fig. 3). It is important to note that synthesis of all PGs by micromass culture homogenates is severely inhibited by indomethacin which in a large number of systems inhibits the cyclo-oxygenase enzyme that catalyses the formation of the endoperoxide precursors of PGs from arachidonic acid (Table 3).

In stage-25 subridge mesoderm cultures, in which cartilage differentiation occurs more rapidly, PG production by micromass culture homogenates from endogenous substrates also reaches a maximum at the onset of overt chondrogenesis,

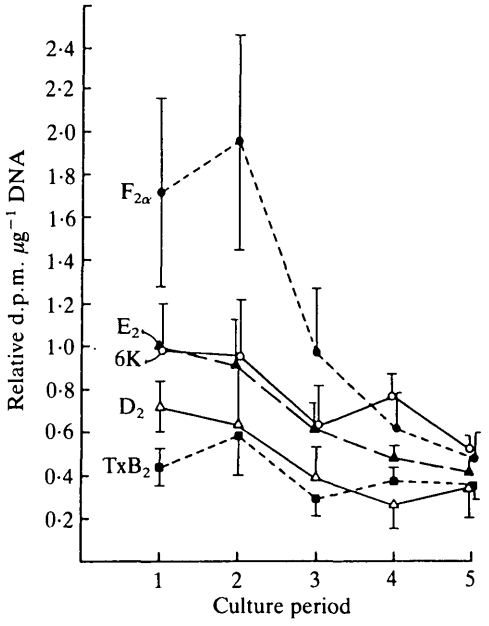

Fig. 2. Amount of various [^{3}H]arachidonic-acid-labelled metabolites produced at various times during the micromass culture of cells of the distal subridge mesoderm of stage-25 wing buds. At 12 h intervals throughout the 3 days of culture, cells were prelabelled with [^{3}H]arachidonic acid for 12 h, and then incubated for an additional 12 h to allow labelled metabolites to be released into the medium. Culture period 1 in the graph represents prelabelling with [^{3}H]arachidonic acid from 0–12 h followed by a chase from 12–24 h; culture period 2, 12–24 prelabelling, 24–36 h chase; culture period 3, 24–36 h prelabelling, 36–48 h chase; culture period 4, 36–48 h prelabelling, 48–60 h chase; culture period 5, 48–60 h prelabelling, 60–72 h chase. In order to validly compare data obtained from six separate experiments done over the course of two years, the data from each experiment were normalized as follows: The amount (d.p.m. μg^{-1} DNA) of each of the labelled metabolites produced during each period of culture was divided by the amount (d.p.m. μg^{-1} DNA) of PGE_2 produced during culture period 1 in that experiment (= relative d.p.m. μg^{-1} DNA). Each value thus represents the mean of six determinations ± S.E.M. $PGF_{2\alpha}$ ($F_{2\alpha}$, ●----●); PGE_2 (E_2, ▲---▲); PGD_2 (D_2, △——△); 6-keto-$PGF_{1\alpha}$ (6K, ○——○); TxB_2 (■----■).

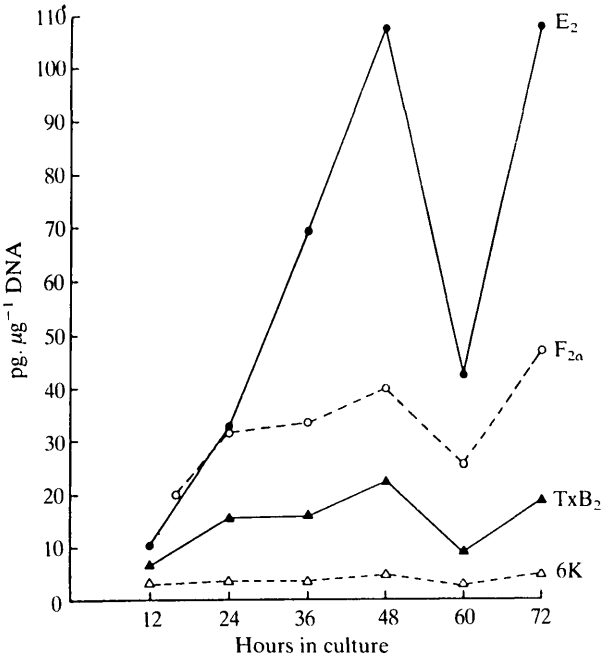

Fig. 3. Amount of various prostaglandins synthesized from endogenous substrates by stage-23/24 whole limb micromass culture homogenates during 1 h incubations at various times following the initiation of culture as determined by radioimmunoassay. PGE_2 (E_2, ●——●); $PGF_{2\alpha}$ ($F_{2\alpha}$, ○----○); TxB_2 (▲——▲); 6-keto-$PGF_{1\alpha}$ (6K, △----△).

i.e. at 36 h (Fig. 4). At that time PGE_2 and TxA_2 production is three to five times greater than at all other periods of culture examined with about twice as much PGE_2 synthesized as TxA_2 (Fig. 4). The amount of $PGF_{2\alpha}$ synthesized by 36 h micromass culture homogenates was not determined, but at all other times the amount of $PGF_{2\alpha}$ synthesized was about equal to the amount of PGE_2 produced. Following overt chondrogenesis (i.e. at 48 and 60 h), synthesis of PGE_2 and TxA_2 by stage-25 micromass culture homogenates sharply declines (Fig. 4). As in stage-

Table 3. *Effect of indomethacin on the synthesis of prostaglandins from endogenous substrates by 16 h stage-23/24 whole limb micromass culture homogenates*

	pg μg^{-1} DNA		
Prostanoid	control	indomethacin (50μm)	Percent inhibition
PGE_2	17·5	1·0	94
$PGF_{2\alpha}$	20·1	3·8	81
TxB_2	3·4	ND*	100
6-Keto-$PGF_{1\alpha}$	2·0	0·6	70

* Not detectable.

23/24 whole limb cultures, prostacyclin (i.e. 6-keto-$PGF_{1\alpha}$) is produced at low levels during all periods of culture.

Thus our radioimmunological results essentially confirm our radiometric TLC analyses in indicating that PG production is highest during periods of culture which coincide with the onset of chondrogenesis in both stage-23/24 whole limb and stage-25 subridge mesoderm micromass cultures. It is noteworthy that the amount of PGE_2 produced at the onset of chondrogenesis by stage-23/24 whole limb micromass culture homogenates is about twice the amount produced at the onset of chondrogenesis by stage-25 subridge mesoderm micromass culture homogenates. This quantitative difference undoubtedly reflects to some extent the fact that a considerable amount of non-chondrogenic tissue differentiates in the stage-23/24 whole limb cultures, while little, if any, non-chondrogenic tissue forms in the stage-25 subridge cultures (see Discussion). It is also noteworthy that our immunological analysis indicates that PGE_2 is the predominant PG produced by micromass culture homogenates from endogenous substrates during a 1 h incubation at the onset of chondrogenesis, while our radiometric TLC analyses indicate that [^{3}H]arachidonic acid metabolites that comigrate with $PGF_{2\alpha}$ are the predominant PGs accumulated during that time. This qualitative difference undoubtedly reflects to some extent the metabolism of parent PG compounds

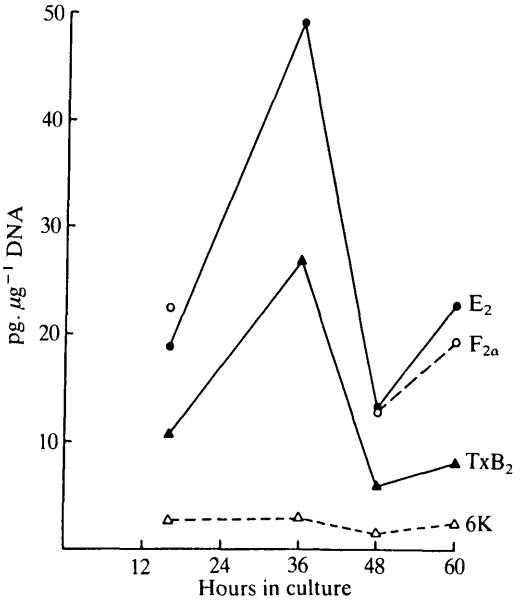

Fig. 4. Amount of various prostaglandins synthesized from endogenous substrates by stage-25 subridge mesoderm micromass culture homogenates during 1 h incubations at various times following the initiation of culture as determined by radioimmunoassay. PGE_2 (E_2, ●——●); $PGF_{2\alpha}$ ($F_{2\alpha}$, ○----○); TxB_2 (▲——▲); 6-keto-$PGF_{1\alpha}$ (6K, △----△).

during the extensive (12 h) labelling and postlabelling incubation periods utilized in our TLC analyses (see above and Discussion).

DISCUSSION

A variety of previous studies have implicated cAMP and prostaglandins in the regulation of limb cartilage differentiation (see Introduction). The results of our combined radiometric thin layer chromatographic and radioimmunological analyses demonstrate that limb mesenchymal cells undergoing chondrogenic differentiation *in vitro* do indeed synthesize several prostaglandins (PGs) including PGE_2, $PGF_{2\alpha}$, prostacyclin, thromboxane A_2, and PGD_2. Furthermore, the results indicate that PG synthesis is at a maximum during periods of culture coinciding with the onset of chondrogenesis as characterized by the formation of extensive prechondrogenic cellular aggregates and their initial conversion into cartilage nodules. It is of considerable interest that the period of maximum prostaglandin synthesis correlates temporally with the transient increase in cAMP content that occurs at the onset of chondrogenesis in micromass culture (Solursh *et al.* 1979; Ho *et al.* 1982; Biddulph *et al.* 1984). The temporal correlations between increased PG production, cAMP accumulation, and the onset of chondrogenesis are consistent with our previous suggestion that PGs produced during the condensation or aggregation phase of chondrogenesis may act as local modulators of cAMP accumulation (Kosher & Walker, 1983; Gay & Kosher, 1984; Kosher & Gay, 1985). In this regard, we have previously demonstrated that the stimulatory effect of PGE_2 on *in vitro* chondrogenesis is potentiated by phosphodiesterase inhibitors (Kosher & Walker, 1983; Gay & Kosher, 1984). Moreover, limb mesenchymal cells do indeed possess adenylate-cyclase-coupled receptors for several PGs including PGE_2 and prostacyclin (Parker *et al.* 1981; Ballard & Biddulph, 1983; Kosher & Gay, 1985), and the relative effectiveness of various PGs stimulating cAMP accumulation in limb mesenchymal cells directly reflects the relative potencies of these same molecules in stimulating *in vitro* chondrogenesis (Kosher & Gay, 1985).

Our results indicate that virtually all PGs examined are produced in relatively high amounts at the onset of chondrogenesis. The predominant [^{3}H]arachidonic-acid-labelled metabolites detected by radiometric TLC during this period are compounds that comigrate with $PGF_{2\alpha}$, whereas PGE_2 is by far the predominant PG synthesized from endogenous substrates by micromass culture homogenates during 1 h incubations throughout the onset of chondrogenesis. This qualitative discrepancy undoubtedly reflects, at least to some extent, metabolism of parent PG compounds during the extensive (12 h) labelling and postlabelling incubation periods utilized in our TLC analyses. The primary PGs produced by virtually all cells undergo quite rapid metabolism and interconversions into compounds which do not necessarily comigrate with the parent PG that was initially synthesized (Samuelsson *et al.* 1978; Hall & Behrman, 1982; Granstorm & Kindahel, 1983). In fact, one of the major metabolites of PGE_2 is $PGF_{2\alpha}$ or compounds which

comigrate with $PGF_{2\alpha}$ on TLC (Hàll & Behrman, 1982; Granstrom & Kindahl, 1983). In view of our radioimmunological results, it is quite conceivable that much of the $PGF_{2\alpha}$ detected in our radiometric TLC analyses resulted from the metabolism of PGE_2.

PG production attains higher levels and is maintained at higher levels during later periods of culture in micromass cultures prepared from the cells of whole stage-23/24 limb buds than in the cultures prepared from the distal subridge mesoderm of stage-25 wing buds, in which more rapid, extensive, and homogeneous chondrogenic differentiation occurs. This quantitative difference is very likely a reflection of the fact that a considerable amount of non-chondrogenic tissue including myogenic cells differentiates in stage-23/24 whole limb micromass cultures, while little, if any, non-chondrogenic tissue forms in the stage-25 subridge mesoderm micromass cultures (Gay & Kosher, 1984). It is conceivable that PGs are synthesized by differentiating fibroblastic connective tissue cells and/or myoblasts, as well as by chondrogenic cells. It has, in fact, been proposed that PGs may play an important rôle in myogenic differentiation (Zalin, 1977). These observations also emphasize the importance of using culture systems in which uniform and homogeneous differentiation occurs when attempting to analyse the molecular events associated with cartilage differentiation.

It is noteworthy that Chepenik *et al.* (1984) have previously shown by thin layer chromatography that high-density cultures of whole stage-24 limbs are capable of producing several prostaglandin-like molecules when they are subjected to mechanical stimulation. Biddulph *et al.* (1984) have recently shown the presence of PGE_2 in the medium of micromass cultures of whole stage-23/24 limb buds and in whole limb buds.

In summary, the results of our present study indicating a temporal correlation between PG production and the initiation of chondrogenesis *in vitro* are consistent with previous investigations implicating PGs in the regulation of chondrogenesis. In this regard, indomethacin and several other inhibitors of endogenous PG synthesis inhibit *in vitro* limb chondrogenesis (Chepenik *et al.* 1984; Kosher, Rodgers, Guiliano & Gay, 1985), and inhibitory effect of these agents can be greatly attenuated by adding exogenous PGE_2 along with them (Kosher *et al.* 1985).

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