The influence of insulin, cAMP and the calcium ionophore X 537 A on the growth of the cartilage anlagen of limb buds in vitro

H.-J. Merker and Th. Günther

Anatomisches Institut und Institut für Molekularbiologie und Biochemie, Freie Universität Berlin, (D-1000 Berlin-West 33), 27 December 1978

Summary. Limb buds of 11-day-old mouse embryos were cultured for 6 days with insulin, dibutyryl cAMP and X 537 A. The cartilage anlage was reduced by insulin and enlarged by dibutyryl cAMP and X 537 A. The effects are due to changes in the amount of intercellular substance.

Characteristic changes in the content of cyclic nucleotides occur during cell proliferation in vitro, and they probably play a role in growth regulation[1-3]. An increase in the intracellular content of cAMP or addition of cAMP inhibits the growth of a wide variety of cell types. In accordance with this, the cAMP content increases if growth is inhibited at high cell density. Addition of cAMP to fibroblast and chondroblast cultures causes an increased synthesis and secretion of proteoglycans (PG) or glycosaminoglycans (GAG)[4-7] and collagen[1,8].

Proliferation of blastemal cells and the synthesis of PG and collagen represent an important step during chondrogenesis and osteogenesis. We therefore studied the influence of dibutyryl cAMP, insulin and the Ca-ionophore X 537 A on the cartilage development of cultured limb buds from 11-day-old mouse embryos. These substances change intracellular cAMP and Ca^{2+} concentrations.

Materials and methods. Limb buds of 11-day-old mouse embryos (strain NMRI) were cultured for 6 days in a Trowell culture modified according to Aydelotte and Kochhar[9] and Neubert et al.[10]. The following substances were added to the medium for the entire culture period at the concentrations: insulin: 1, 2 or 4 IU per ml medium, cAMP: 1 mM (as dibutyryl cAMP, Boehringer, Mannheim), X 537 A (Hoffmann-La Roche, Basel): 10^{-6} to 5×10^{-5} M. The planimetric estimation of cartilage areas was carried out with the Quantimet (Kontron) after staining with methylene blue. Fixation for inspection by electron microscope was carried out with 3% paraformaldehyde plus 3% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.4. Postfixation with 1% OsO_4 in a similar buffer was followed by embedding in Mikropal. Sectioning was carried out using LKB and Reichert microtomes. A Zeiss EM 10 and a Siemens 101 electron microscope were used.

Results. cAMP, insulin and the Ca-ionophore X 537 A influenced the size of the planimetrically measured cartilage anlagen without causing any changes in the pattern of the cartilage skeleton. Increasing the concentration of insulin reduced the area of the cartilage anlagen by up to 45% of that of controls (table). After addition of 1 mM dibutyryl cAMP the cartilage anlagen were 40% larger than those of the controls.

X 537 A also changed the development of cartilage anlagen dose-dependently. Low concentrations led to an increase in cartilage area. Higher doses (3 and 5×10^{-5} M) inhibited the planimetrically measurable cartilage growth and necroses occurred. Therefore these toxic concentrations are not included in the table.

Increasing the Ca^{2+} concentration above the normal value had no effect. The increase in extracellular Ca^{2+} concentration after the addition of 5 mM $CaCl_2$ in the presence of X 537 A had the same effects as X 537 A in the presence of physiological Ca^{2+} concentration. This shows that at physiological extracellular Ca^{2+} concentration, the influx of Ca^{2+} into the chondroblasts is saturated in the presence or absence of X 537 A.

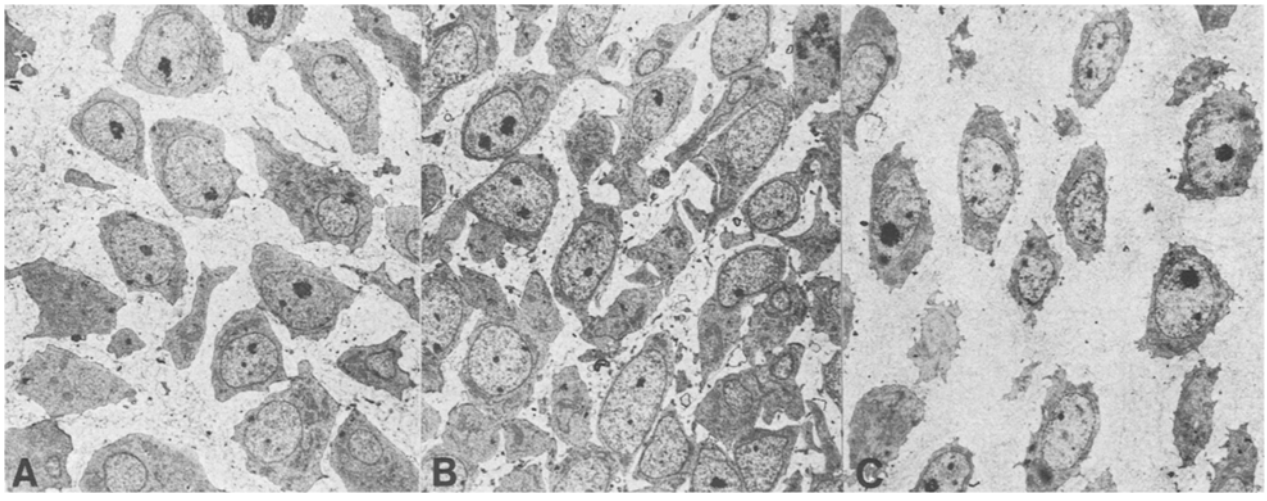
Our light and electron microscopical investigations have shown that the increase and the decrease in the size of cartilage anlagen were due to a change in the amount of intercellular substance (ICS). Under the influence of insulin the cartilage anlagen became smaller and the cartilage cells were located close together (figure, B) so that the amount of ICS per cell and per cartilage anlage was reduced. In the cartilage anlagen, which had enlarged under the influence of cAMP, cartilage cells were situated so far apart (figure, C) that a relative and - owing to the increase in the size of the cartilage anlagen - also absolute increase in ICS occurred. The same as for cAMP was observed in cartilage anlagen that had enlarged after addition of X 537 A. A more detailed quantitative analysis is not possible because the total number of chondrocytes per cartilage anlage under the various experimental conditions was not measurable with the methods used.

The structure of the cells and of the collagen filaments, their packing pattern and course and the appearance of PG-granules with their radiating filaments did not show any alterations after the addition of insulin, cAMP and X 537 A. Therefore it can be concluded that the ICS is altered

Effect of insulin, dibutyryl cAMP and X 537 A on size and fraction of ICS of cartilage anlagen in 11-day-old limb buds after a 6-day culture period

	Dosage	N	Size of cartilage anlagen (mm^2)	p	Fraction of ICS (%)
Control	-	32	0.69 ± 0.13		63 ± 5
Insulin	1 IU/ml	16	0.64 ± 0.21	-	*
	2 IU/ml	16	0.58 ± 0.14	-	*
	4 IU/ml	16	0.38 ± 0.10	<0.001	51 ± 7
Dibutyryl cAMP	1 mM	16	0.96 ± 0.22	<0.01	72 ± 8
X 537 A	1×10^{-6} M	16	0.98 ± 0.20	<0.01	77 ± 7
	5×10^{-6} M	16	0.90 ± 0.15	<0.01	*
$CaCl_2$	1 mM	16	0.66 ± 0.12	-	*
	3 mM	16	0.68 ± 0.10	-	*
	5 mM	16	0.60 ± 0.13	-	*
	7 mM	16	0.61 ± 0.20	-	*
X 537 A + $CaCl_2$	1×10^{-5} M + 5 mM	16	0.85 ± 0.13	<0.01	*

Mean ± SD, p, significance * Not measured.



Cartilage (diaphyseal region of radius) from 11-day-old limb buds after a 6-day culture period. A: Untreated control. B: Treated with 4 IU/ml insulin. C: Treated with 1 mM dibutyryl cAMP. ×2000.

only quantitatively and not qualitatively by these substances.

Discussion. The results obtained from investigations on connective tissue cells in vitro may serve to elucidate the altered collagen and PG/GAG synthesis. According to these findings, the addition of dibutyryl cAMP inhibits the growtx of cells[1-3] and increases their synthesis of collagen[1,8] and PG/GAG[4-7].

The same effect produced by X 537 A can be attributed to an increased influx of Ca^{2+} into the cells[11] and an increase in cytosolic Ca^{2+} concentration[12,13]. An increase in the cytosolic Ca^{2+} concentration, normally amounting to 10^{-7} to 10^{-6} M[13], activates adenylate cyclase[13-16] and phosphodiesterase[16], which preferably splits cGMP[16,17], leading to an increase in cAMP[18] and a decrease in cGMP concentration within the cell[17]. Thus with X 537 A the same mechanism may function as in the presence of dibutyryl cAMP.

On the other hand cAMP induces an increase in cell permeability for K^+ and Ca^{2+}[19] and might thus cause an increase in cytosolic Ca^{2+} concentration, which would correspond to the effect of the Ca-ionophore. According to several investigators[13,20-22], cytosolic Ca^{2+} concentration itself has regulatory functions in the metabolism of connective cells. No statement can be made about the quantitative role of cAMP and Ca^{2+}, or about a synergistic effect of cAMP and Ca^{2+} on the regulation of collagen and PG/GAG synthesis.

The inhibiting and toxic effect of high doses of X 537 A is possibly due to the fact that at high concentrations this substance is incorporated into mitochondrial membranes and then inhibits the oxidative energy metabolism[18].

The mode of action of insulin might be due to its function as a growth factor, thus possibly leading to a higher number of cells in the cartilage anlagen. Since cell growth correlates with low cAMP concentrations, insulin may secondarily induce a lower cAMP concentration and thus a lower rate of collagen and PG/GAG synthesis. On the other hand, insulin may primarily decrease cAMP concentration and may thus elevate the growth rate. Since insulin does not reduce the concentration of cAMP in all cell types (e.g. diaphragm[23]), its mechanism has to be established for chondroblasts. Moreover, insulin increases intracellular K^+ concentration by activating Na^+-K^+-ATPase[24,25]. X 537 A reduces intracellular K^+ concentration[18] by being not absolutely specific for divalent cations[11]. At elevated cytosolic Ca^{2+} concentrations, the intracellular K^+ concentration is reduced by increased passive permeability[26]. Thus, it is possible that changes in the intracellular K^+ concentration are also involved in the regulation of growth and synthesis of ICS in cartilage anlagen.

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