THE DIFFERENTIAL SENSITIVITY OF CULTURED CHICK MESODERMAL CELLS TO ACTINOMYCIN D

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ABSTRACT

Cells were isolated from the somite mesoderm and from the unsegmented (presomite) mesoderm of early chick embryos and exposed to actinomycin D in single cell culture. Actinomycin D inhibited proliferation in cell cultures derived from the unsegmented mesoderm, although the same concentrations of this antibiotic did not inhibit cultures derived from the somite mesoderm. This differential sensitivity parallels the regionally specific necrosis and degeneration observed in the unsegmented mesoderm of intact chick embryos exposed to actinomycin D. In culture, both cell types exhibited approximately the same permeability to labeled actinomycin D and showed comparable inhibition of RNA, DNA, and protein syntheses in the presence of the antibiotic. However, freshly isolated mesodermal cells from the somite region had a higher content of RNA than did cells from the unsegmented region, and the somite cells maintained a higher rate of macromolecular synthesis in untreated cultures.

INTRODUCTION

The injection of actinomycin D into the yolk sacs of 2–3 day chick embryos resulted in a high incidence of abnormalities in the posterior trunk and limbs (1). The first histological defects detected in these treated embryos involved cellular necrosis and degeneration in the trunk mesoderm posterior to the somites (2). This regionally specific inhibition was also observed with chick embryos of 11–13 somites cultured in vitro for 48 hr on an egg-agar medium containing actinomycin D (3). The anterior regions of these embryos were morphologically normal and contained levels of RNA, DNA, and protein nitrogen comparable to untreated embryos. These observations indicated that the posterior region of the chick embryo, and specifically the trunk mesoderm that has not yet segmented to form somites, is sensitive to concentrations of actinomycin D that do not inhibit the more anterior regions of the embryo, including the somite mesoderm.

Two alternatives have been proposed to account for the sensitivity of the unsegmented axial mesoderm (3, 4). First, actinomycin D may be unequally distributed in the embryo so that the presomite mesoderm contains a greater concentration of the inhibitor. This could result from differences in the structure of tissues, the proximity of tissues to the circulatory system, or differences in cell permeability. On the other hand, the differential sensitivity could reflect alterations in the metabolic activities of the mesodermal cells during the transition from the unsegmented to the segmented state.

In order to evaluate these alternatives, cells

from the somite mesoderm and those from unsegmented mesoderm were exposed to actinomycin D in single cell culture. This procedure made it possible to expose all cells equally to the antibiotic and to eliminate differences between the two cell types which depend on their location in the embryo.

MATERIALS AND METHODS

Fertile White Leghorn eggs were incubated for 40 hr at 37.5°C. The embryos were removed from the eggs into sterile chick Ringer's solution, and those containing 11–13 somites were selected for experimentation.

After removing the portion of the embryo anterior to the first somite, the remaining trunk was separated into segmented and unsegmented regions. The tissue from each region was then incubated for 1–3 hr at room temperature in Hanks balanced salt solution (5) containing 0.25% trypsin-pancreatin (Nutritional Biochemicals Corporation, Cleveland, Ohio) so that the mesoderm could be separated from the ectoderm, notochord, and neural tube. The resulting blocks of mesodermal tissue were then incubated at room temperature in 0.25% trypsin-pancreatin in a calcium-, magnesium-free salt solution (6). After approximately 1 hr, the tissues could be dissociated into single cells by gentle pipetting. A similar quantity of cells was obtained from each region. 50 embryos were required to obtain 10^6 cells of each type. The mesodermal cells were collected from the trypsin solution by centrifugation and were cultured in 50 mm watch glasses or 60 mm glass Petri dishes at 37°C under 95% air, 5% CO_2. The basal medium was a modified Eagle's (7) containing sodium bicarbonate, 1.60 grams per liter. Cell number was determined by hemocytometer counts.

Because it was not possible to maintain cultures in Eagle's medium alone, horse serum and embryo extract were used as media supplements. Cultures of 25,000 cells in 0.5 ml did not require embryo extract and proliferated in medium containing as little as 0.5% horse serum. By supplementing the basal medium with 10% horse serum and 10% embryo extract, the volume and density of the cultures could be increased to 10^6 cells in 4 ml. Higher levels of horse serum were inhibitory. These same requirements for media supplementation were observed when medium 199 (8) was used instead of Eagle's as the basal medium. The horse serum was obtained from Microbiological Associates, Inc., Bethesda, Md. and the embryo extract was prepared by a 1:1 dilution of the supernatant from the homogenization of 12-day chick embryos with Hanks balanced salt solution.

The culture conditions described above permitted the maintenance of chick mesodermal cells in primary culture for up to 72 hr. Cell number increased in the cultures at a constant rate over the entire culture period. The 0.5 ml cultures doubled in cell number in 36 hr; the 4 ml cultures required 72 hr to double. The cells were viable at the end of the culture period as judged by the nigrosine dye exclusion test (9), and there were no indications of cell degeneration in light microscope examinations of living and fixed cells. No attempt was made to maintain the cultures longer than 72 hr.

Uncultured mesodermal cells were analysed to determine their content of DNA, RNA, and protein. Aliquots of 250,000 cells were washed to remove the trypsin, and cold trichloroacetic acid was added to a final concentration of 5%. After overnight precipitation at 4°C, the lipids were removed with 70°C chloroform-ethanol. RNA was extracted in 10% perchloric acid for 18 hr (10) and DNA was extracted at 70°C with 5% perchloric acid for 20 min (11). Quantitation was done colorimetrically: orcinol for RNA (12), diphenylamine for DNA (13), and the Lowry method for protein (14). Bovine serum albumin (Nutritional Biochemicals), yeast RNA (Calbiochem, Los Angeles, Calif.), and calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) were used as standards.

Radioactive precursors of protein (L-leucine-^{14}C, UL [Schwarz Bioresearch Inc., Orangeburg, N.Y.], 200 mc/mmole) and nucleic acids (uridine-2-^{14}C [Schwarz], 25 mc/mmole) were administered to 3 ml cultures for 3 hr at a concentration of 0.25 μc/ml. The pulse was terminated by chilling the cultures to 5°C. Because the fragility of the cells after culture precluded pelleting them by centrifugation, Millipore filters (24 mm, SM, 5μ) were employed to collect the cells. After six rinses with 5 ml of chick Ringer's, the cells were fixed to the filters in 95% ethanol overnight. The cells were extracted by placing the filters sequentially in 5% cold trichloroacetic acid, 10% cold perchloric acid, and 5% hot perchloric acid. After each extraction the filters were rinsed with ethanol, dried, and the radioactivity was measured in a Nuclear-Chicago gas-flow low-background planchet counter (Nuclear-Chicago Corporation, Des Plaines, Ill.).

^{14}C-actinomycin D (3000 cpm/μg) was administered to 4 ml cultures of 10^6 cells at a concentration of 5 μg/ml. After 6 hr, the cells were collected on Millipore filters and rinsed with chick Ringer's until the wash contained no measurable radioactivity. The cells were dried on the filters and radioactivity was determined in the planchet counter.

Actinomycin D was generously provided through the courtesy of Dr. Laurent Michaud of Merck, Sharp and Dohme, and the ^{14}C-labeled actinomycin D was the generous gift of Dr. Edward Reich of The Rockefeller University.

RESULTS

Although untreated cultures of cells obtained from chick axial mesoderm had the same growth charac-

TABLE I

Response of Somite and Unsegmented Mesoderm Cells to Actinomycin D in Culture

Medium with addition of					cells/culture	
HS	EE	Act. D	Initial inoculum	Culture time	Somite mesoderm	Unsegm. mesoderm
per cent	*per cent*	*μg/ml*	*cells/culture*	*hr*		
10	10	None	25,000	24	41,500	42,500
10	10	1	25,000	24	42,000	37,500
10	10	10	25,000	24	38,500	33,000
10	10	100	25,000	24	38,000	24,000
10	0	None	25,000	24	42,000	45,000
10	0	50	25,000	24	42,000	34,000
10	0	100	25,000	24	35,000	21,000
0.5	0	None	25,000	36	54,000	52,000
0.5	0	50	25,000	36	54,000	33,000
10	10	None	250,000	36	371,000	348,000
10	10	50	250,000	36	376,000	248,000
10	10	None	250,000	72	450,000	410,000
10	10	50	250,000	72	435,000	240,000

Cultures of 25,000 cells had a 0.5 ml volume; cultures of 250,000 cells had a 4 ml volume. Abbreviations: *HS* = horse serum; *EE* = embryo extract; *Act. D* = actinomycin D.

teristics whether the cells were obtained from segmented or from unsegmented regions, the two cell types responded differently to treatment with actinomycin D (Table I). In the presence of up to 100 μg/ml of the antibiotic, the proliferation of cells in cultures derived from the segmented mesoderm was only slightly inhibited (0–17%). At the same concentrations of actinomycin D, the increase in cell number of the cultures from the unsegmented mesoderm was completely inhibited. This differential response was examined under various conditions of medium supplementation and with several culture volumes and cell densities. Although the concentration of actinomycin D necessary to effect complete inhibition of the unsegmented mesodermal cells varied as culture conditions were altered, in all cases it was possible to find a dosage of the antibiotic which selectively inhibited the presomite mesodermal cells.

Quantitative analyses were performed on uncultured mesodermal cells from the segmented and unsegmented regions of the embryonic trunk (Table II). The two cell types did not differ significantly in either DNA or protein content. However, the segmented mesodermal cells contained 25% more RNA than did the cells of the unsegmented mesoderm. This difference was highly significant.

Protein and nucleic acid syntheses were also examined in the two cell types by using radioactive precursors. L-leucine-^{14}C was used for incorporation into protein, and uridine-2-^{14}C was used for incorporation into both RNA and DNA. Uridine was originally administered to the cultures to monitor RNA synthesis. However, it was noted that there was substantial incorporation of uridine into both nucleic acids while thymidine proved to be a poor DNA precursor in this system. This unusually efficient utilization of uridine as a precursor for DNA by the early chick embryo has also been reported in the radioautographic studies of Emanuelsson (15) and was suggested by Roth and Askew (16) who observed low levels of thymidine kinase in the early embryo.

The results of the experiments with radioactive precursors are presented in Table III. After 4 hr of culture, control cultures of somite and unsegmented mesodermal cells did not differ in their ability to incorporate leucine into protein or uridine into RNA and DNA. After 36 hr of culture, both cell types showed reduced ability to incorporate these precursors. However, this reduc-

TABLE II

Content of DNA, RNA, and Protein in Somite and Unsegmented Mesodermal Cells

Cell type	μg RNA / 250,000 cells	μg DNA / 250,000 cells	μg protein / 250,000 cells
Somite	8.27 ± 0.39	1.44 ± 0.07	91.23 ± 3.77
Unsegm.	6.65 ± 0.25	1.43 ± 0.07	86.07 ± 3.80

Each value is the average of 10 determinations from two separate preparations. The difference in RNA is highly significant ($p < .01$). The values for DNA and protein are not significantly different ($p > .3$).

TABLE III

Mean Values of Incorporation of ^{14}C-Leucine into Protein and Uridine-2-^{14}C into DNA and RNA by Mesodermal Cells in Culture

Cell type	Act. D	Time of culture before pulse	cpm/10^6 cells RNA	DNA	Protein	No. of determinations
		hr				
somite	−	4	2542	5084	12039	(6–7)
somite	+	4	16	16	3456	(2–6)
unsegm.	−	4	3373	4637	11348	(6–7)
unsegm.	+	4	24	22	3612	(2–5)
somite	−	36	150	346	6730	(4–6)
somite	+	36	3	1	19	(2–3)
unsegm.	−	36	63	114	1373	(4–6)
unsegm.	+	36	0	3	27	(3)

The duration of the pulse was 3 hr; actinomycin D was employed at a concentration of 50 μg/ml; the isotopes were added to a final concentration of 0.25 μg/ml.

tion was more severe in the cells of the unsegmented mesoderm. Under actinomycin D treatment, the two cell lines showed the same patterns of inhibition. Actinomycin D (50 μg/ml) reduced leucine incorporation to about 30% within the first 4 hr while the incorporation of uridine into RNA and DNA was reduced to less than 1% of the untreated control. After 36 hr, the incorporation of both leucine and uridine was inhibited below 1%.

In order to determine the relative permeability of mesodermal cells to actinomycin D, cultures of cells from the somite and unsegmented regions were exposed for 6 hr to ^{14}C-actinomycin D (Table IV). Although this study was limited to only two cultures for each cell type, the incorporation of radioactive antibiotic was as high in the insensitive cell line as it was in the sensitive cells of the unsegmented mesoderm.

TABLE IV

The Uptake of ^{14}C-Actinomycin D by Mesodermal Cells in Culture

Cell type	Culture No. 1	Culture No. 2	Average
	cpm	*cpm*	*cpm*
Somite	1273	1919	1596
Unsegm.	1332	1388	1360

Cultures of 10^6 cells were exposed for 6 hr to ^{14}C-actinomycin D at a concentration of 5 μg/ml.

DISCUSSION

In the intact chick embryo, *in ovo* or in culture, the unsegmented (presomite) axial mesoderm is highly sensitive to actinomycin D when compared to the other regions of the embryo (1, 2, 3). This specificity is potentially useful as a tool to investi-

gate the events of mesoderm differentiation. However, a necessary first step in interpreting this phenomenon was to establish whether this differential sensitivity was a result of an unequal distribution of the antibiotic in the embryo or whether it reflected a basic cellular difference between somite and presomite mesoderm.

From the experiments described in this paper it can be concluded that the presomite mesoderm retains its sensitivity to actinomycin D in cell culture. Thus the sensitivity reflects a property inherent in these cells and independent of the location or structure of the mesodermal tissue in the intact embryo. Because somite cells remain relatively insensitive to actinomycin D in culture, the change in sensitivity is conferred on the mesodermal cells during the process of segmentation.

It can also be concluded from the experiments with ^{14}C-actinomycin D that the sensitivity of the unsegmented mesodermal cells is not a result of preferential uptake of the antibiotic. These data do not give any information on the binding of actinomycin within the cell, nor do they necessarily eliminate the possibility that there is a distribution gradient in the intact embryo. However, because the two cell types respond differently to actinomycin D under conditions where the exposure and uptake of the antibiotic are equivalent, such a distribution gradient, if it exists, could only enhance the basic cellular difference between presomite and somite mesoderm.

The observation that actinomycin completely inhibits the increase in cell number in the presomite cultures can be interpreted several ways. The antibiotic may inhibit division of these cells and thus prevent culture growth. The same effect would be achieved if cell division continued, but was balanced by an increase in cell mortality. It is also possible to find dosages of actinomycin which result in partial inhibition of the growth of presomite cultures (Table I). It is not clear whether this represents an incomplete inhibition of all the cells or a complete inhibition of a fraction of the cells. Concentrations of actinomycin D higher than those found to selectively inhibit the presomite cells completely prevented increases in cell number in cultures of both type of cells (J. A. Piper, and N. W. Klein. Unpublished data).

The results of the isotopic incorporation studies and biochemical analyses reveal that after segmentation the mesodermal cells contain 25% more RNA and are able to maintain higher synthetic activities in culture. Although these changes may be significant in the process of somite differentiation, it is not clear from the information available at this time whether they are the basis for the differential sensitivity.

The nature of the cellular difference between the mesoderm of the unsegmented plate and the other tissues of the chick embryo is not resolved at this time. If it is assumed that the lack of increase in cell number in actinomycin-treated cells of the unsegmented mesoderm reflects an absence of cell proliferation rather than increased cytotoxicity, then the effect of the antibiotic on these cells is to block some stage in the cell cycle and prevent cell division. In contrast, the somite cells are able to complete at least one cell cycle, resulting in cytokinesis. It may well be that the two cell lines differ in their requirements for biosynthesis preceding mitosis. The observation that the cells from the unsegmented mesoderm are less capable of maintaining their synthetic activities in the later hours of culture also suggests that these cells may have more extensive metabolic requirements than do the cells of the somite mesoderm.

Actinomycin D-insensitive processes are not uncommon in embryological systems. The development of amphibian and sea urchin eggs proceeds normally through cleavage and up to gastrulation in high concentrations of this inhibitor (17, 18). In vitro studies on a variety of developing tissues show a period of actinomycin insensitivity following the initial phases of differentiation. Some of these systems include: zymogen production by pancreas rudiments (19), hemoglobin synthesis in chick red blood cells (20), kidney tubulogenesis (21), crystalline production in embryonic and regenerating lenses (22, 23), and differentiation of muscle cells in culture (24). These phenomena are explained by evoking the presence of a stable messenger RNA in the insensitive cells which permits the continuation of protein synthesis in the absence of additional RNA synthesis. Such a premise does not seem indicated in the system under consideration in this paper, however, for total protein synthesis is as severely inhibited in the insensitive cell line as it is in the sensitive cells. A definite conclusion on this point would require an examination of the synthesis of specific proteins.

The cells of embryonic chick mesoderm are able to proliferate in the presence of concentrations of actinomycin ten times those usually necessary to completely inhibit division of cells from established mammalian lines (25–27). In this respect the meso-

dermal cells more closely resemble the cells of the sea urchin and amphibian blastulae (17, 18). Recent reports have indicated that actinomycin D has effects on cultured cells in addition to its well-known inhibition of DNA-dependent RNA synthesis. Studies with prelabeled Hela cells suggested that RNA synthesized immediately before actinomycin D administration is rapidly degraded in the presence of the antibiotic (28). Reports of depressed levels of ATP and oxygen consumption (29), mitochondrial hyperplasia (30), and reversal of synthetic inhibition by glycolytic substrates (31) have suggested that alterations in oxidative phosphorylation and energy production might result from actinomycin D treatment. These side effects, whether immediate consequences of actinomycin treatment or secondary results of the inhibition of RNA synthesis, must be considered in evaluating the action of this antibiotic on the chick embryo, especially in view of the high dosages used in this study.

It is hoped that further investigation of the basis for the differential sensitivity between segmented and unsegmented mesoderm will give insights into the cellular changes that accompany this early stage of somite differentiation.

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