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DNA Synthesis and Differentiation in Embryonic Kidney Mesenchyme in vitro

Abstract. *Mitosis accompanies the differentiation of embryonic metanephrogenic mesenchyme in vitro. Inhibition of mitosis by 5-fluorodeoxyuridine, but not by x-rays, is associated with inhibition of differentiation. The difference may be due to the effect of 5-fluorodeoxyuridine on DNA synthesis.*

Inhibition of DNA synthesis by 5-fluorodeoxyuridine reportedly inhibits differentiation (elongation) of nondividing plant cells (*1*). 5-Fluorodeoxyuridine has an inhibitory effect on the differentiation of embryonic metanephrogenic mesenchyme in vitro, which may also be independent of cell division.

5-Fluorodeoxyuridine (FUDR) specifically inhibits thymidylate synthetase (*2*, *3*), thus blocking DNA synthesis (*4–6*) and, consequently, inhibiting mitosis (*6*) and cell division (*4*, *5*). To determine whether inhibition of differentiation by FUDR was caused by inhibition of DNA synthesis or mitosis or both, I tried to distinguish between these alternatives by comparing the effect of mitotic inhibition by x-rays with the effect of treatment with FUDR.

Mesenchyme from the metanephric kidney of the mouse embryo differentiates in vitro in response to inductive stimulation from embryonic dorsal spinal cord (*7*). By 24 hours of culture most of the mesenchymal tissue is arranged in whorls, containing many cells in mitosis, which, on further culture, gradually develop into tubules.

Metanephric kidneys and dorsal spinal cords were isolated from 11-day-old mouse embryos [C×C_3H(HeAu)] by methods previously described (*7*). The kidney rudiments were incubated for 4 to 5 minutes in a 3 percent trypsin solution containing 3 parts trypsin (1:250 Difco) to one part pancreatin (Viokase, Viobin Corp.) dissolved in calcium- and magnesium-free Tyrode's solution. Mesenchyme was then separated from the ureteric bud by flushing the kidney into and out of a micropipette (*7*). Mesenchyme from two to three kidney rudiments was fused to a piece of dorsal spinal cord, cultured in a filter-well assembly (*8*) in a Grobstein dish containing 1.5 ml of medium, and maintained in a humidified incubator gassed with 5 percent CO_2 in air at 37°C. 5-Fluorodeoxyuridine and $10^{-4}M$ uridine were added to medium containing 1 percent horse serum and 50 units of penicillin-streptomycin per milliliter in Eagle's basal medium (Medium A.) Irradiated tissues were cultured in medium containing 3 percent embryo extract, 10 percent horse serum and 50 units of penicillin-streptomycin per milliliter in Eagle's basal medium (Medium B). Both media support differentiation, but growth is more extensive and survival longer in the more richly supplemented medium. Mesenchyme to be irradiated was maintained in a culture dish containing 0.5 ml of medium placed in a glass container filled with 5 percent CO_2 in air. X-rays were administered at 140 kv (peak) and 5 ma with an inherent filtration of ½ mm of aluminum at a target distance of 30 cm in single-dose treatments of 1500 r at a rate of 55 r/min. Dosage was monitored with a Victoreen dosimeter.

To determine the effects of FUDR and x-ray treatments on DNA synthesis, incorporation of tritiated deoxycytidine was studied by autoradiography. Tissues were incubated for 1 hour in medium containing 10 μc of tritiated deoxycytidine per milliliter (Schwarz BioResearch, Inc., 2.4 c/mmole), washed twice in medium without labeled precursor, fixed in Carnoy's for 40 minutes, embedded, and sectioned at 4 μ. Alternating strips of the paraffin ribbon containing four or five sections of tissue were placed on three slides. Tissues on two slides were hydrolyzed for 4 hours at 37°C: one in 0.3 mg of deoxyribonuclease (Worthington) per milliliter in $4 \times 10^{-3}M$ $MgSO_4$, *p*H 7, the other in 0.3 mg of ribonuclease (Worthington) per milliliter, *p*H 7. The third slide was untreated. All slides were coated with Kodak AR-10 stripping film at 25°C, developed in D19b developer after 2 weeks of exposure, and stained with Delafield's hematoxylin. Treatment with ribonuclease caused no significant alteration in the pattern of labeling, whereas treatment with deoxyribonuclease removed virtually all the labeled material. For routine histological preparations, the cultures were fixed in Carnoy's, sectioned at 5 μ, and stained with Delafield's hematoxylin and eosin.

The effect of increasing concentrations of FUDR on differentiation is summarized in Table 1, experiment 1. Cultures grown in $10^{-6}M$ FUDR are indistinguishable from controls. Those grown in $10^{-5}M$ FUDR are retarded in development, and the tubules which are formed are smaller, are not as well shaped as those in controls, and contain fewer mitotic figures. Cultures survive for at least 24 hours in $10^{-4}M$ FUDR but show no signs of differentiation. No mitotic figures were observed at 24 hours.

Autoradiographs prepared from tissues cultured in medium containing $10^{-4}M$ FUDR to which tritiated deoxycytidine was added during the 4th to 5th hour of incubation show that treatment with FUDR greatly reduces, but never completely abolishes, uptake of labeled precursor in both the mesenchyme and dorsal spinal cord. It was impossible to quantitate the extent of the inhibition because there was no discernible regularity in the distribution of labeled cells throughout the cultures. Resistance to FUDR (*3*) and synthesis of "DNA-like material" during thymidineless growth (*4*) has been reported for other tissues, but the mechanisms involved are not clear.

The inhibitory effect of FUDR on differentiation was shown to be reversible in two ways. Cultures were grown in medium containing $10^{-4}M$ FUDR to which $10^{-4}M$ thymidine was added. Differentiation proceeded normally in this medium (Table 1, experiment 2). All media supplemented with FUDR contained $10^{-4}M$ uridine. Under modified culture conditions, in which differentiation can be inhibited with lower concentrations of FUDR, a hundredfold increase in the concentration of uridine

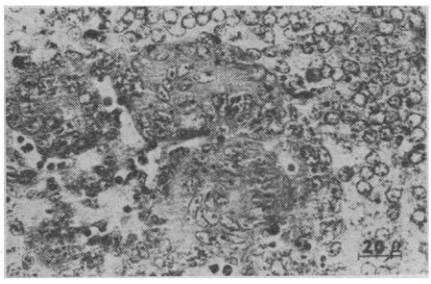

Fig. 1. Tubules formed after culture for 20 hours in the presence of $10^{-4}M$ FUDR followed by 20 hours in control medium B. Surrounding tissue is dorsal spinal cord.

also did not reverse inhibition (*9*). The effect of FUDR on differentiation thus probably reflects a primary interference with the synthesis of DNA rather than with that of RNA. One cannot exclude the possibility, however, that thymidylate deficiency affects differentiation by some unknown means. That no simple reaction relates thymidine with induction is indicated by the failure of mesenchyme to differentiate when it is cultured in medium containing $10^{-4}M$ thymidine in the absence of dorsal spinal cord.

In a second test (Table 1, experiment 3), the fused tissues were cultured in $10^{-4}M$ FUDR for 20 hours and then transferred to the more enriched, regular medium. Tubules, which differed from controls in their frequent lack of distinct lumina and in their lack of nuclei oriented radially with respect to their long axes, began to form after a 20-hour delay (Fig. 1). Cells were somewhat enlarged, and mitotic figures were rare. These latter observations are consistent with reports of lost reproductive capacity (*4, 5*) and subsequent cellular enlargement caused by unbalanced growth (*4*) following similar periods of incubation in FUDR. Recovery of differentiative potential in the absence of cell division would imply that inhibition of differentiation by FUDR is not caused by inhibition of mitosis, loss of viability, or genic imbalance in the progeny of those cells exposed to FUDR during the period of DNA synthesis (*10*).

Table 1. Effect of 5-fluorodeoxyuridine on differentiation. The number of cultures which formed whorls or tubules out of the number of cultures tested in three trials of each experiment is given as the positive (No.)/total (No.).

Composition of test medium				Positive (No.)
Medium A	Medium B	FUDR	Thymidine	Total (No.)
		Experiment 1		
+	0	$10^{-6}M$	0	9/9
+	0	$10^{-5}M$	0	9/9
+	0	$10^{-4}M$	0	0/18
+	0	0	0	6/6
		Experiment 2		
+	0	$10^{-4}M$	$10^{-4}M$	7/8
+	0	0	0	5/5
+	0	$10^{-4}M$	0	0/6
		Experiment 3		
+	+*	$10^{-4}M$	0	8/8
+	+†	0	0	7/7
+	0	$10^{-4}M$	0	0/6

* Fused mesenchyme and dorsal spinal cord were cultured for 20 hours in medium A containing $10^{-4}M$ FUDR, then transferred to medium B for 20 hours. † The fused tissues were cultured in medium A for 20 hours then transferred to medium B for 20 hours.

In test cultures, irradiated mesenchyme from five kidney rudiments was fused to a piece of dorsal spinal cord. As a control for the irradiation treatment, five irradiated mesenchymal pieces were fused to a piece of ventral spinal cord, which lacks inductive capability (*7*). As a positive control, nonirradiated mesenchyme from two kidney rudiments was fused to a piece of dorsal spinal cord. Histological preparations showed that whorls had formed in the irradiated mesenchyme fused to dorsal spinal cord cultured for 24 hours but not in that fused to ventral spinal cord. After 48 hours, the first group contained a few small whorls, with no discernible mitotic figures, surrounded by tissue debris, while the mesenchyme of the second group was completely necrotic.

Mitotic indexes were calculated in cultures fixed at 24 hours. Since mitotic figures are few and nonrandomly distributed in whorls formed by irradiated mesenchyme, I could examine virtually the entire whorl population by scanning every other section of the culture. Six cultures containing 52 whorls were examined in this way and the mean mitotic index was then calculated from the number of cells in mitosis per 100 cells of a whorl. The mitotic index of whorls in control cultures was based on counts of representative sections of 14 whorls, each totaling at least 200 cells, in four cultures. The mean mitotic index of whorls formed by nonirradiated mesenchyme was 0.6 ± 0.3; that of whorls formed by irradiated mesenchyme was 4.7 ± 0.5. The difference is statistically significant ($P < 0.001$). It should be noted, however, that the inhibition of mitosis observed at this time need not reflect a similar inhibition during the preceeding interval (*11*).

Autoradiographs of cultures containing irradiated and nonirradiated mesenchyme which had been exposed to tritiated deoxycytidine during the 23rd to 24th hour of culture had labeled cells in both mesenchyme and dorsal spinal cord. Labeling indexes and grain densities per cell were calculated for one test and one control culture. Whorls with large diameters in cross section, possibly containing a somewhat lower proportion of labeled cells, were excluded from the analysis because their boundaries are difficult to distinguish. Such sections are relatively rare in irradiated tissue, where the tendency is to form small whorls, but are more frequent in controls. Labeling indexes were calculated as the mean of the proportion of labeled cells per section of a whorl multiplied by 100. A count of 96 labeled cells (7 whorl sections) in irradiated mesenchyme yielded a labeling index of 68 ± 6; 102 labeled cells (6 whorl sections) in nonirradiated mesenchyme gave an index of 90 ± 7, which is in essential agreement with that determined by other workers measuring incorporation of tritiated thymidine by tubules (*12*). A rough measure, probably the upper limit, of the degree of dissociation of mitosis and DNA synthesis in irradiated tissue is indicated by comparison of the labeling and mitotic indexes; the labeling index is 68 ± 6; the mean mitotic index, 0.6 ± 0.3. Therefore one mitosis per 113 labeled cells was seen.

The mean value of grains per cell was based on a count of 96 cells in whorls of irradiated mesenchyme and of 74 cells in nonirradiated mesenchyme. The rates of incorporation are similar, the mean grain count being 42 ± 2 grains per cell over irradiated tissue and 37 ± 1 grains per cell over nonirradiated tissue. Some incorporation in irradiated cells may reflect repair of broken chromosomes, but this is unlikely to be the major cause since the proportion of the irradiated cells which were labeled was smaller than that of the controls.

If these inferences concerning the dis-

sociation of DNA synthesis and mitosis are correct, they suggest that DNA synthesis is a requirement, independent of mitosis, for certain sequences in the differentiation of tubules. The evidence does not indicate whether such a requirement would involve doubling of the DNA content, synthesis at specific loci of the genome (*13*), or metabolic turnover of DNA (*14*).

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Crystal and Molecular Structure of Acetylselenocholine Iodide

Abstract. *The structure of acetylselenocholine iodide has been determined by x-ray crystallographic analysis. The molecule is in the* trans *conformation about the C—C bond of the choline residue. This conformation appears to explain the molecule's inability to give a positive cholinergic response when added to an electroplax preparation.*

Recent studies (*1*) with an isolated single cell electroplax preparation showed that acetylcholine and acetylselenocholine (ASeCh) have markedly different pharmacological activities. The seleno derivative was found to be inactive in a very wide concentration range on this cholinergic receptor site. Though a striking difference in the reactivity exists for these compounds on this cholinergic receptor, the two molecules are hydrolyzed at similar rates by acetylcholinesterase (*2*). In an effort to obtain structural information about ASeCh and also the structural requirements of the active sites of acetylcholinesterase and the electroplax preparation, the crystal structure of ASeCh iodide was investigated.

Single crystals of the compound were supplied by H. G. Mautner (Yale University). We measured the following crystallographic data: $\mathbf{a} = 7.923 \pm 0.006$ Å; $\mathbf{b} = 11.83 \pm 0.01$ Å; $\mathbf{c} = 13.22 \pm 0.01$ Å; space group, $P\,2_12_12_1$; ρ (measured by flotation) = 1.801 g/cm^3; ρ (calculated assuming four molecules in the unit sell) = 1.802 g/cm^3.

Three-dimensional intensity data were measured with a General Electric XRD-5 diffractometer by the stationary counter-stationary crystal technique with Mo$K\alpha$ radiation. Balanced filters were used. The intensities were corrected for the Lorentz and polarization factors. No absorption corrections were made. In total, 570 reflections had peak intensities greater than twice their calculated standard deviations [errors of the S.D.'s computed by method of Evans (*3*)]. The coordinates of the atoms comprising the molecule were readily obtained from three-dimensional Patterson and Fourier syntheses. The positional and thermal parameters of the molecule were refined by block diagonal least squares analysis. The final R value (the usual discrepancy index) was 0.055 for the observed data.

In discussing the pertinent features of the molecule it is most interesting to make a comparison with the very recently reported structure of acetylcholine bromide (*4*). The most dramatic difference between the two molecules is the conformation about the C—C bond of the choline moiety. The torsion angle (ϕ_{cc} defined as the dihedral angle between the projection of the C—N$^+$ and the C—Se or C—O bonds) is *gauche* in acetylcholine, that is, in the neighborhood of 60°, and *trans* in the present structure, 179° (see Fig. 1). These represent the two possible staggered conformations possible about the C—C bond. Sundaralingam (*5*) has indicated that in N$^+$—C—C—O systems the *gauche* conformation is always the preferred one. The reason for the *trans* conformation in this structure is not as yet completely clear, but it is most probably due to the larger van der Waals radius of the selenium atom, 2.0 Å as compared to oxygen's 1.4 Å. Presently, work on the thio-derivative is being carried out to determine the influence of the van der Waals radius on the conformation.

It is interesting to note that a staggered conformation exists about the C(4)—N bond. This conformation was also observed in acetylcholine.

The C(2)—Se and C(3)—Se bond lengths are 1.82 Å and 2.18 Å (± 0.05 Å), respectively. These distances deviate significantly from the C—Se distance of 1.977 Å found in the electron diffraction study on dimethyl selenide (*6*). In acetylcholine it was also observed that the bond lengths about the ester oxygen deviated in the same manner from the commonly accepted C—O single bond length. It was noted in the acetylcholine report that the partial double bond character in the carboxyl carbon to oxygen bond is consistent with the mechanism of hy-

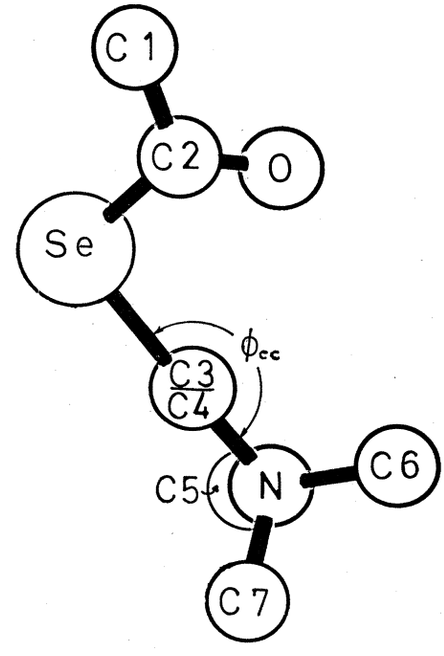

Fig. 1. Projection of molecule down the C(3)—C(4) bond.