Effect of Cigarette Smoke on Rabbit Corneal Cells in Vitro

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Cigarette smoke strongly affects rabbit corneal cells in vitro. The cells from hypercholesteremic rabbits are damaged more severely by cigarette smoke than the cells from normocholesteremic rabbits.

In vitro, the effect increases with increasing concentration of cigarette smoke. This is reflected by the decrease in the number of positive cultures, by the delay of the initial outgrowth of cells, by the depression of growth value, by the distortion of growth curves, by the morphologic alterations, and by the depression of enzymatic activities of corneal epithelial cells.

Quantitative reaction of rabbit corneal cells to cigarette smoke is similar to that of rabbit’s aortic cells. The qualitative response of corneal epithelial cells to cigarette smoke was far less pronounced than that of aortic endothelial cells.

The cornea and the aorta have many common structural characteristics. Rabbit corneal epithelial cells and aortic endothelial cells grown in tissue cultures have many morphologic similarities according to Adachi and Pollak.1,2 Analogous response of cornea and aorta to lipoids, to lipoprotein fractions, and to hyperlipemic serum has been reported by Waters3-6 and by Silver et al.7

The effect of nicotine, of cigarette smoke condensate, and of cigarette smoke on rabbit aortic cells in vitro has been investigated by Kokubu and Pollak8 by Koide and Pollak,9 and by Kasai and Pollak,10 respectively. We now studied the effect of cigarette smoke on rabbit corneal cells in vitro.

MATERIALS AND METHODS

To obtain various concentrations of cigarette smoke, a series of five test tubes, each with 10 ml of nutrient media, were connected as a “train.” U-shaped segments of glass tubing provided the connections between the test tubes. A

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lighted cigarette was connected to the short arm of the first U-tubing; the long arm of this tubing, fitted with fritted glass, reached into the nutrient in the first tube. The tubing leading from the last test tube was connected to a constant-suction pump. The tubes were agitated during preparation of dilutions. The unit of cigarette smoke, CS, was defined as that amount of smoke from an untipped, commercially denicotinized cigarette led into 1 ml of nutrient media. The last of the five test tubes contained 1/50 of smoke from one cigarette, or CS/50. By progressive dilution with nutrient CS/100, CS/200, and CS/400 were obtained. The reaction of the treated nutrient was adjusted to pH 7.2–7.4 using 1.4% sodium bicarbonate solution. This procedure has been used by Kasai and Pollak10 for aortic cells and therefore it was used in the study of corneal cells. No attention was paid to possible fractional dissolution of gases.

The tissue culture methods used for corneal cells were the same as those described for aortic cells by Kokubu and Pollak.11 The corneal explants studied numbered 300. Equal numbers of explants were used in each of 10 in vitro series. The histochemical methods applied in this study were described and pertinent references cited in a report by Adachi and Pollak.2

The blood cholesterol of one-half of the rabbits ranged between 45 and 90 mg per 100 ml, that of the other half was between 600 and 1,600 mg per 100 ml.

**RESULTS**

The data presented in Fig. 1 are shown in the same manner as used for aortic cells by Kasai and Pollak.10 The curves represent the averages. The ranges of variations for each group of experiments were narrow: The curves for minimum and maximum deviations were so close to the average curves that reproduction of the former two could be omitted.

The number of positive corneal cultures decreased with increasing amounts of cigarette smoke in the nutrient. The effect of CS/400 and CS/200 was slight, while that of CS/100 and CS/50 was marked. Under higher concentrations of CS the corneal explants from hypercholesteremic rabbits yielded significantly fewer positive cultures than those explants from normocholesteremic rabbits.

The earliest outgrowth of cells was delayed by the addition of smoke to the nutrient. Yet, not even where CS had markedly decreased the number of successful cultures, the earliest outgrowth was delayed more than 24 hours after the inoculation in the nutrient.

The growth curves for corneal cells from normo- and hypercholesteremic rabbits were affected by cigarette smoke, even in the lowest concentration used. The curves began to decline after 5 days under CS/400 and CS/200, and were the same for explants from normo- and hypercholesteremic rabbits. With CS/100 and CS/50 the curves began to decline on the 5th day for explants from normocholesteremic rabbits, and on the 3rd day for explants from hyperchole-
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Fig. 1. Effect of cigarette smoke (CS) on corneal cells in vitro from normo- (N) and hypercholesteremic rabbits (H). CS was added to the nutrient before inoculation at various concentrations.

0: no smoke. CS/400, CS/200, CS/100, CS/50: smoke from 1/400, 1/200, 1/100, 1/50 of a cigarette respectively. G.V. (growth values): 0, no new cells; 1, 1–50 new cells; 2, 50–100 new cells; 3, 101–1,000 new cells; 4, 0.1 x 10⁴–1 x 10⁴ new cells; 5, 1 x 10⁴–6 x 10⁴ new cells; 6, 6 x 10⁴–1 x 10⁵ new cells; 7, over 10⁵ new cells.

Numbers in the upper right corner in each graph represent the percentage of positive cultures.

steremic rabbits. The slope of the curve was slightly exaggerated for cells from the corneae of hypercholesteremic rabbits.

Morphologic alterations of corneal cells exposed to cigarette smoke were marked. In lower CS concentrations, shrinkage of the cytoplasm and thickening of the nuclear membrane were the dominant features. Often, the nuclear membrane became indistinct. Under higher CS concentrations, pyknosis became prominent. Vacuolization was hardly ever seen. There was no difference in morphologic alterations between explants from normo- and hypercholesteremic rabbits.

Nine enzymes were studied histochemically. Photomicrographs depicting the effect of CS/200 and CS/50 are not reproduced in this report since the trend of decreased enzymatic activity with increasing CS concentration is clearly apparent from photographs of corneal epithelial cells not exposed to smoke, (O), exposed to CS/400, and exposed to CS/100. Malic dehydrogenase was not affected by CS/400, and alkaline phosphatase and monoamine oxidase were only slightly depressed by CS/400, but all three enzymes were depressed by CS/100. Acid phosphatase, adenosine triphosphatase, lactic and succinic dehydrogenases, and cytochrome oxidase were readily depressed by CS/400; higher concentrations of CS only exaggerated the effect of the additive. With decreased cytoplasmic activity, there was often a tendency toward increase in nuclear enzymatic reactivity. The amounts of lipase were negligible in controls (0) and also under various CS concentrations.
DISCUSSION

The effect of cigarette smoke on rabbit corneal cells in vitro reflects the strength of CS added to the nutrient media before inoculation. With increasing CS concentration, the number of positive cultures decreased, the lag phase was delayed, the growth values declined faster, and the growth curves became distorted, flattened, and shortened. Statistical analysis of the experimental results is not feasible. Each growth value used for plotting of the curves represents a wide logarithmic range. Corneal cells from rabbits with hypercholesteremia were, in all respects, more vulnerable to CS than corneal cells from normocholesteremic rabbits. The severity of morphologic alterations increased with the amount of CS in the nutrient. This was also true for enzymatic activity of corneal epithelial cells. No significance could be attached to the observation that malic dehydrogenase, acid phosphatase, and monoamine oxidase resisted the toxic influence of CS longer than the other enzyme. In evaluating changes in the size of cells, the nucleo-cytoplasmic ratio, or the cytoplasmic granularity, one must bear in mind the physiologic variations. Similarly, the intensity of enzyme stains and the intracellular distribution of specially stained matter may vary from one culture to another and within the same cell population. This phenomenon was pronounced for alkaline phosphatase of corneal epithelial cells whether or not these cells were exposed to cigarette smoke. Statistical analysis is impossible as well as unnecessary as long as no significance is claimed for.

Comparison of the reaction of corneal cells in vitro with the reaction of aortic cells in vitro suggests a similar quantitative response of cells from two sources. Under analogous conditions, i.e., the same strength of cigarette smoke, both types of explants yielded a comparable number of successful cultures and similar growth patterns. Quantitative response to CS was not the same: Vacuolization of the cytoplasm which is one of the outstanding features of the reaction of aortic cells to toxins was rarely seen in corneal cells. While other regressive changes caused by the addition of CS to the nutrient were comparable, the degree of alterations of aortic endothelial cells was far severer than that of corneal epithelial cells exposed to the same dose of cigarette smoke. Greater vulnerability of aortic cells in vitro may be ascribed to the fact that during life these cells are exposed to internal forces, whereas, corneal cells exposed to extraneous factors.

The greater vulnerability of corneal cells from hypercholesteremic rabbits is of interest. Greater damage to aortic cells by nicotine in hypercholesteremic rabbits has been reported by Kokubu and Pollak,8 greater damage to aortic cells of such rabbits by cigarette smoke condensate has been recorded by Koide and Pollak.9 Exaggerated effect of cigarette smoke on aortic cells from hypercholesteremic rabbits has been noted by Kasai and Pollak.10 Hypercholesteremia
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weakens the resistance of rabbit tissue to toxic substances such as nicotine and particulate matter of cigarette smoke.

References


Legends for Figs. 2-10

Fig. 2–10. Enzymatic reactions of corneal epithelial cells grown in nutrient media without additive (0)-upper, with CS/400-middle, and with CS/100-lowest photograph. All photomicrographs from 4 day old cultures; all, ×1000.

Fig. 2. Acid phosphatase.
O: Brown precipitate throughout the cytoplasm and close to the nuclear membrane. Cytoplasmic activity stronger than nuclear reaction.
CS/400: Cytoplasmic precipitate coarser. Decreased cytoplasmic activity.
CS/100: Exaggeration of trend seen under CS/400.

Fig. 3. Alkaline phosphatase.
O: Variation from cell to cell. The weaker the cytoplasmic reaction in a cell, the stronger the nuclear reaction.
CS/400: Similar to control, 0.
CS/100: Markedly decreased activity. Some nuclei stain much darker than the majority.
Fig. 4. Adenosine triphosphatase.
0: Brown to black precipitate throughout the cytoplasm. Little staining of the nuclei.
CS/400: Fewer and lighter staining granules in the cytoplasm.
CS/100: Coarser precipitate. Smudging of granules and of the nuclear membrane.

Fig. 5. Lipase.
0: Negligible activity.
CS/400: Same as control, 0.
CS/100: Foamy cytoplasm but no change in activity.

Fig. 6. Monoamine oxidase.
0: Coarse cytoplasmic granules in juxtanuclear areas; fewer granules in the rest of the cytoplasm. No granules in the nuclei.
CS/400: Similar to control, 0. Decrease in perinuclear aggregates.

Fig. 7. Lactic dehydrogenase.
0: Fine, purple precipitate throughout the cytoplasm, with perinuclear predilection. No granules in the nuclei.
CS/400: Cytoplasmic reaction weaker, granules coarser. Nuclear granules present.

Fig. 8. Malic dehydrogenase.
0: Purple cytoplasmic granules. Juxtanuclear predilection. No granules in the nuclei.
CS/400: Same as control, 0.
CS/100: Markedly weakened activity. Sparse cytoplasmic granules.

Fig. 9. Succinic dehydrogenase.
0: Coarse, purple or blue-black precipitate throughout the cell. Cytoplasmic activity stronger than nuclear reaction.
CS/400: Staining pattern similar to control, 0, but much weaker.
CS/100: Still further lessened reaction. Coarser granules.

Fig. 10. Cytochrome oxidase.
0: Heavy perinuclear aggregates of positive granules. Scattered granules in the rest of the cytoplasm and in the nuclei.
CS/400: Weaker reaction. Coarser granules.
CS/100: Marked weakening of the reaction in the periphery of the cytoplasm. Increased perinuclear staining and nuclear reaction.
Fig. 4.

Fig. 5.
Fig. 10.