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The effect of nicotine on cultured cells of vascular origin

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Summary. Monolayers of endothelial, smooth muscle and fibroblastic cells of healthy porcine, bovine or human fetal origin were treated with 10^{-4} to 10^{-9} M final concentrations of nicotine. The effect was registered as changes in the synthesis and polimerization of the cytoskeleton. The silver and gold impregnation method produced anisotropy of the synthetic granules and of the final polymers of microtubules and filaments under physiological conditions as revealed by polarization microscopy. Since the orientation of the cells was inhomogenous in the cultures, the organization of the orientation was expressed as the sum of alternative diagonal and orthogonal measuring of anisotropy by a computerized microraster morphometry system joined to an OPTON cytophotometer. The 8-day-old control and treated cultures were also examined by electronmicroscopy.

Nicotine stimulated the synthesis and polimerization of the cytoskeletal protein. This phenomenon is evident in smooth muscle cells, and partly also in endothelium. Fibroblasts were not influenced by the doses of nicotine tested.

Key words: Nicotine – Aortic cell cultures – Cytoskeleton – Computerized morphometry

Introduction

Following the Surgeon General's Report in 1964 many epidemiological investigations have confirmed that in addition to a high level of blood cholesterol and hypertension, cigarette smoking is also a risk factor in atherosclerosis and coronary heart disease [14, 9]. This was shown by "The Multiple Risk Factor Intervention Trial" (MRFIT), an American-Canadian cooperative study in which a follow up of about 13 thousand patients was carried

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out from 1974 to 1981 [19]. Evidence has been presented that moderately elevated carboxyhaemoglobin levels are associated with various changes in the cardiovascular system [1]. Despite the fact that among the noxious ingredients of tobacco smoke, nicotine has the strongest pharmacological effect [18] only a few studies are available to date in support of the hypothesis that chronic nicotine action may be involved in the development of atherosclerosis]10]. Recently a complex effect of nicotine on plasma lipoproteins has been reported [9, 2].

In the present study we have examined the effects of low concentrations of nicotine on the cytoskeletal system of bovine and porcine endothelial, porcine smooth muscle and human fibroblasts cells. In the concentrations used (10^{-4}–10^{-9} M) nicotine had no general cytotoxic effect on the cultured cells; 10^{-5}–10^{-6} M nicotine concentration is equivalent to the mean serum level in humans 20 min after smoking a cigarette [3, 16]. The endothelial and smooth muscle cells have decisive role in maintaining the integrity of the arterial wall. Increased permeability of endothelial cells, changes in the intercellular material transport, the stimulation of secretory and cell migration activity are all considered to be early signs of cell damage [6, 15, 12, 11, 13]. It is well known that the cytoskeleton plays an important role in intra- and intercellular material transport, in cell migration and possibly also in the secretory and receptor induced endocytotic activities of the cells. The latter maintain a higher than osmotic or diffusional level of directed material transport as part of their function [4, 8, 5, 17]. The purpose of the present work was to assess the abundance of the cytoskeletal microtubular-filamentous system as a cellular indicator of an active protein synthesis and polymerisation.

Materials and methods

The measurements were carried out on coverslip cultures, using the following passages and lines. Passage 5 of bovine aortic endothelial cell culture BAEC-782 (Budapest aortic endothelial cell line). Passage 4 of miniature-pig aortic endothelial cell culture BAEC-785 (Budapest aortic endothelial cell line). Passage 4 of mini-pig aortic smooth muscle cell culture BASC-792 (Budapest aortic smooth msucle cell line), Passage 4 of human fetal fibroblast 2F.

All cell lines were prepared from healthy donors and maintained in this laboratory. For the experiments coverslip cultures were prepared in Leighton tubes using 10% fetal calf serum containing Dulbecco's MEM.

Treatment with nicotine. 10^{-3} M sterile stock solution was prepared from nicotine in PBS. Further dilutions of the stock solution ranging from 10^{-4} M to 10^{-9} M were prepared in Dulbecco's MEM containing 10% fetal Calf Serum.

On the 2nd day of cultivation the nutrient medium was replaced by the medium containing different final concentrations of nicotine. The cultures were incubated for 8 days. During this time medium exchange was performed on the 3th and 6th days using an adequate freshly prepared medium for each group.

The coverslip cultures were fixed in 4% formaldehyde for further processing.

Impregnations. The gold toning impregnation by Gallyas as modified by Óváry [19] was used to visualize the anisotropy of the cytoskeletal system.

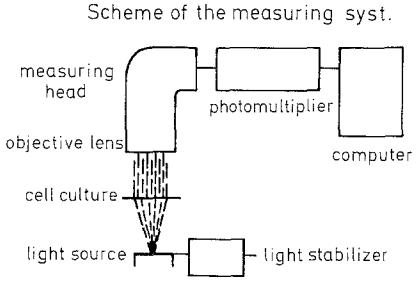
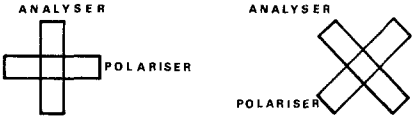


Fig. 1. A gross scheme of the measuring system: the perceptor head is screening the fibers glittering in the visual field, and sends a signal equilvalent to the brightness of the given filament via the photomultiplier to the storing and developing computer



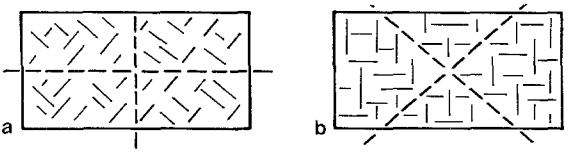


Fig. 2a, b. Analysis of filament orientation
a Orthogonal mode;
b diagonal mode (—direction of brightest filaments)

Morphometry. The preparations were examined by a polarizing microscope. An Ehringhaus measuring compensator was used within the range of 0 to 62, with an objective 16× or 40× and diaphragm 0.05 or 0.1 mm.

The organization of the subcellular structural orientation was examined by alternative orthogonal and diagonal measuring technique using a computerized microraster morphometry system joined to an Opton cytophotometer (Fig. 1). The machine produces a base matrix of columns and rows on the 2.5 × 2.5 μm fields. For each slide the brightest fiber in the proper orientation was selected as the 100 per cent reference of the actual scale. The fields were then scanned first orthogonally then diagonally (Fig. 2). It is evident from the figure that comparison of the results of the two measurements gives information on the preferential orientation of fibers in the given field. The sums of the intensities measured in both orientations in the individual 2.5 × 2.5 μm square fields were averaged usually for about 1,000 fields per slide to obtain a mean total intensity value. For final evaluation the background "noise" was filtered out by defining and appropriate threshold.

Electron microscopy. The cultured bovine aortic endothelial cells were also examined by transmission electron microscopic method.

Results

Direct polarization microscopic inspection did not reveal any conspicuous difference between the preparations. The small but regular differences were detectable only by detailed and careful morphometric analysis. The histograms (Fig. 3) shows the untreated cultures. Each culture was examined in two definite orientations (*vis.* orthogonal and diagonal) relative to a fixed virtual Carthesian coordinate in the microscope. The orientation of filaments is conspicuous in smooth muscle cells. The mean number of diagonal filaments is about 2.5 times higher than that of the orthogonal ones. In the fibroblast cultures similar preference was observed for the diagonal orientation of the filaments; no favoured orientation was found in the endothelium. The latter generally grew radially in small discrete islands. Changes exhibited by the

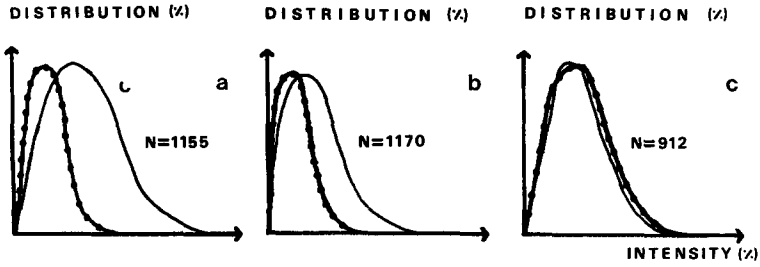


Fig. 3a–c. histogram of filament orientation in different cells **a** Smooth M; **b** fibroblast; **c** endothelium (– = diagonal filaments; -●-●-●- = orthogonal filaments)

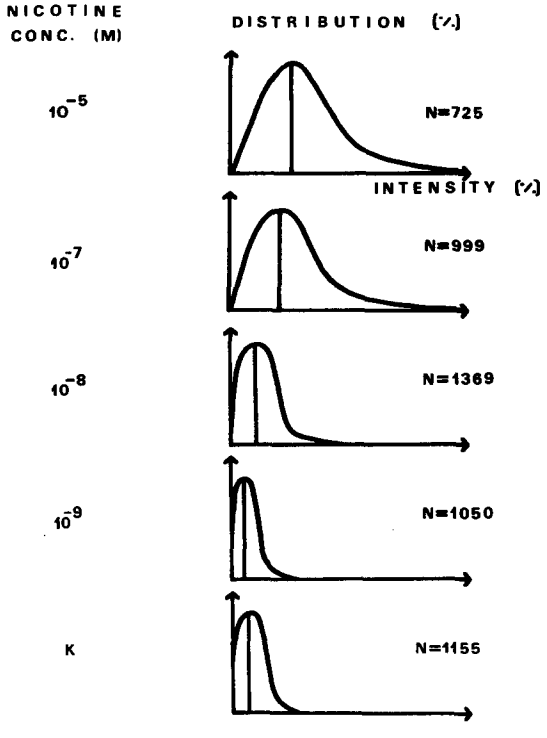


Fig. 4. Dose-response curves of nicotine effect on smooth muscle cells

nicotine treated smooth muscle cell cultures are shown in Fig. 4 were dilution is increasing from the top to the bottom. The number of filaments, expressed as a mean intensity, increased in cultures with increasing concentrations of nicotine. No changes were, however, detectable in the orientation of the filaments. The same relation is shown in Fig. 5. The mean intensities of the unit fields for different slides were normalized to the maximal values actually measured. The mean intensity values for different cultures are comparable only after renormalization because different objectives and diaphragms were needed for each. Regression was calculated from the data. The regression lines for endothelial, smooth muscle and fibroblast cells are shown in Fig. 5. In contrast to the smooth muscle and endothelial cells the increased concentration of nicotine did not result in an increase in the mean polarization intensity in cultured fibroblast.

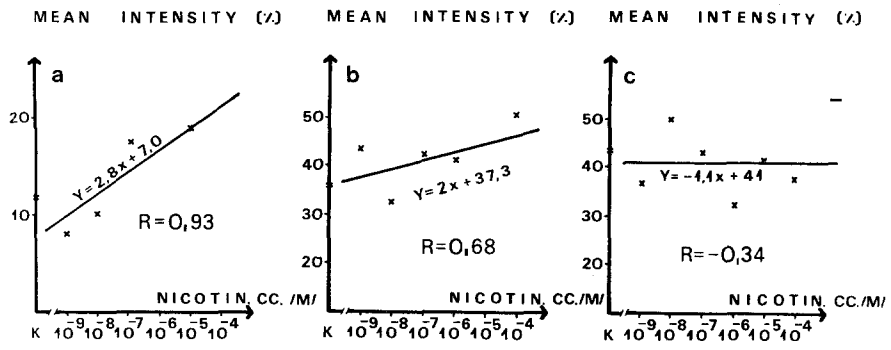


Fig. 5. Effect of nicotine on the quantity of filaments in smooth muscle cells (**a**) endothelial cells (**b**) and fibroblasts (**c**)

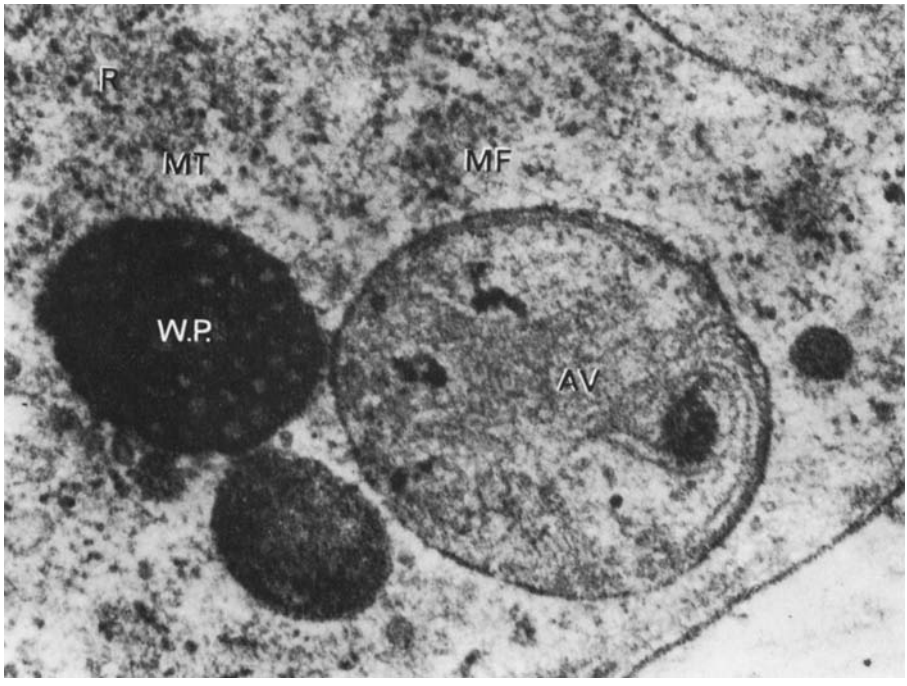


Fig. 6. Part of an untreated, control endothelial cell: Glutaraldehyde fixation. Magnification ×46,800. *W.P.*, Weibel-Palade body; *MT*, microtubules; *MF*, microfilaments; *AV*, autophagic vacuoles; *R*, ribosomes

The electronmicrographs of the untreated bovine endothelial cells showed the usual characteristic features: Weibel-Palade bodies, microfilaments and tubules, free ribosomes and some autophagic vacuoles (Fig. 6). However, many cells treated with 10^{-4} M nicotine showed degeneration and contained prominent concentric membranous arrays (Fig. 7). It should be noted here that these "toxic" effects were seen only in the electronmicrographs of endothelial cells. No electron microscopy was done with the other cell types.

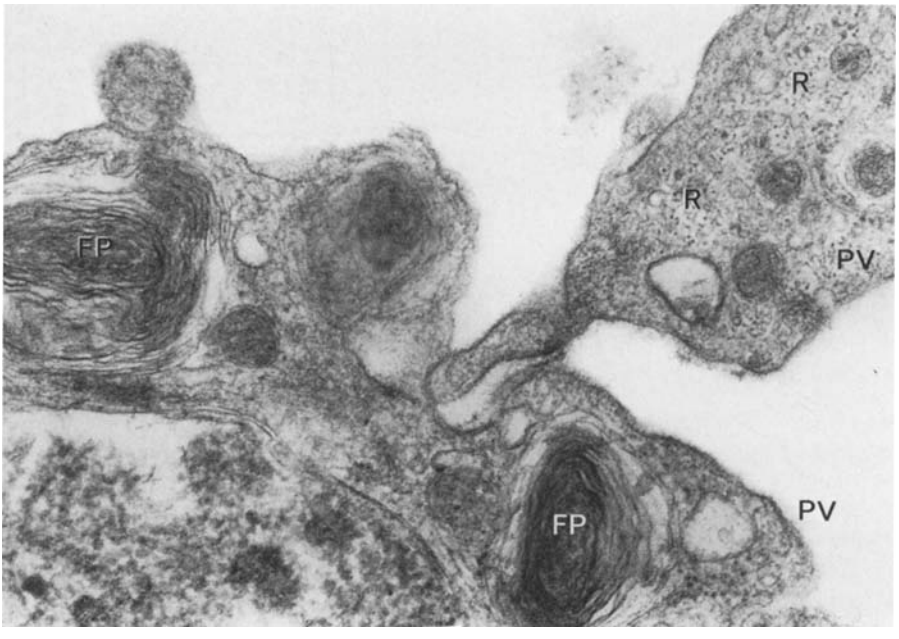


Fig. 7. Bovine endothelial cells treated with 10^{-4} M nicotine Glutaraldehyde fixation. Magnification ×41,400. *FP*, finger print like pattern; *PV*, pinocytotic vesicles; *R*, ribosomes

Discussion

Cultured vascular smooth muscle and endothelial cells and fibroblasts reacted differently to nicotine. It appears that low levels of nicotine stimulate cytoskeletal protein synthesis and polymerization. This phenomenon is evident in smooth muscle cells and it is suggestive also in endothelium. Fibroblasts were found to be indifferent to the amount of nicotine used. Since the sensitivity of the different cells were not the same to nicotine the examination should be extended further to other doses of nicotine and other types of cells. Above a certain concentration nicotine causes the stimulation of the synthesis and polymerization of the microtubular-filamentous system. On further increase of nicotine concentration, the inhibition of the cytoskeletal polymerization is seen first, the protein synthesis is damaged next, and is followed by an ultimate cellular destruction. One may speculate that this kind of change in the cytoskeletal system reflects significant alterations in the cell's function and some of the above changes may represent the first steps of cellular damage leeding ultimately to this formation of an atherosclerotic plaque.

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