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Interferon and tumour necrosis factor production in bovine aorta endothelial cells aging *in vitro*. The influence of culture conditions on age-dependent phenotypic changes

Wytwarzanie interferonu i czynnika martwicy nowotworu w komórkach śródbłonka aorty bydlęcej w czasie ich starzenia się *in vitro*. Wpływ warunków hodowli na zależne od wieku zmiany fenotypowe

SUMMARY

Aging of endothelial cells *in vitro* is connected with several phenotypic changes. In this paper we have examined the interferon (IFN), tumour necrosis factor (TNF) production by aging *in vitro* bovine aorta endothelial cells (BAECs) at different passage levels, cultivated in plastic flasks (two--dimensional cultures). Senescent cells (at passage level 16–32) produced significantly less IFN and significantly more TNF in comparison to cells at 4 passage level. When BAECs cultivated in two-dimensional cultures were transferred into three-dimensional cultures on gelatine sponge discs (Spongostan Special^R) no changes in IFN and TNF production were detected in comparison to two--dimensional cultures. Variations in gene expression dependent on *in vitro* aging but independent on the culture conditions have to be taken into consideration in studies concerning the role of endothelial cells in physiology and pathology.

STRESZCZENIE

Starzenie się komórek śródbłonka *in vitro* wiąże się z wieloma zmianami fenotypowymi. W tej pracy badano wytwarzanie interferonu (IFN) i czynnika martwicy nowotworu (TNF) w starzejących się *in vitro* komórkach śródbłonka aorty bydlęcej (BAECs) hodowanych i pasażowanych w plastykowych butelkach (hodowla płaska, dwuwymiarowa). Komórki stare (na poziomie 16–32 pasażu) wytwarzały znacznie mniej IFN i znacznie więcej TNF niż komórki młode na poziomie 4 pasażu. Gdy BAECs hodowane na płaskiej powierzchni przeniesiono do hodowli przestrzennej na krążkach gąbki żelatynowej (Spongostan Special^R), nie obserwowano zmian w wytwarzaniu IFN i TNF w porównaniu z hodowlą dwuwymiarową. Zmiany w ekspresji genów, które zależą od procesu

starzenia się komórek, lecz nie zależą od warunków ich hodowli, powinny być brane pod uwagę w badaniach dotyczących roli komórek śródbłonka w procesach fizjologicznych i patologii.

Key words: interferon, tumour necrosis factor, endothelium, three-dimensional cultures.

INTRODUCTION

Endothelial cells (EC) are mesenchyme-derived cells that line the inside of all blood vessels. Vascular cells are both: a target for cytokines and a source of cytokines. On exposure to various environmental stimuli, EC undergo profound changes in gene expression and function allowing them to participate in various immunological reactions, especially inflammation (3). Gram-positive and Gram-negative bacteria and their products interact with endothelium and induce cytokine production; this interaction accounts for some of the systemic manifestations of sepsis as well as localised reactions. Especially LPS elicits a spectrum of endothelial responses which are mediated by IL-1 and TNF. Some cytokines as IL-6, IL-8, G-CSF and GM-CSF, produced by endothelium are also inducers of other cytokines (15, 17). Also viruses infect and interact with endothelium and induce cytokine production. Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), influenza and Sendai virus were described to induce IFN-α/β and IL-1 production (17, 19, 24).

EC isolated from large blood vessels such as bovine aorta and human umbilical vein can be propagated in culture as non-transformed cells with a finite life span. EC maintained in standard two-dimensional cell culture have been used to investigate several pathological states such as atherosclerosis, genetic abnormalities, cytokine mediated cell damage and tumour metastasis (3, 6).

EC maintained in standard two-dimensional cell culture tend to lose many of their differentiated phenotypic properties that limit the study of differentiated cell functions *in vitro*. For example, EC differentiation antigen CD34 and vonWillebrand factor are down regulated upon transfer of EC in culture (7). Moreover, aging of EC *in vitro* is connected with significant variations in their ability to produce triglicerides or angiotensin converting enzymes (ACE) (5). The life-span of EC *in vitro* can be increased by treatment with fibroblast growth factor (4, 8–12), especially in the presence of some extracellular matrix components (16). Three-dimensional spheroid model of EC cultivation, useful in study of EC differentiation (14), was also described. Recently biodegradable gelatine sponges are widely used in tissue engineering (13).

Spongostan^{R} (Ferrosan, Denmark), pig skin gelatine foam was successfully used for cultivation of mouse bone marrow and peritoneal macrophages (1),

therefore, it was of interest to cultivate EC on Spongostan^R in three-dimensional cell cultures in order to examine the influence of culture conditions on age--dependent changes in cytokine (interferon and tumour necrosis factor) production ability of EC.

MATERIAL AND METHODS

Cell cultures

Bovine aorta endothelial cells (BAECs) originated from vessels were obtained from freshly slaughtered steers and heifers (n = 5). Vessels were clamped before dissection from the surrounding tissue, excised, rinsed with phosphate buffered saline (PBS) and filled with warm 0.25% trypsin. After 30 min at 37°C endothelial cell suspension was collected and centrifuged (250×g for 10 min). The cell pellet was re-suspended and subsequently cultured in Dulbecco's modified Eagle's medium (DMEM), containing 10% foetal bovine serum (FCS), 100 μg/ml of penicillin, 100 μg/ml streptomycin. The cells were grown in tissue flasks in CO_2 incubator (5% CO_2) at 37°C. Endothelial cell identity (at passage 0) was verified by direct phase contrast microscopy and by indirect immunofluorescence microscopy after staining for factor VIII-related vonWillebrand antigen (Dakopatts, Denmark). Cells were serially passaged by the routine method of trypsinization of grown monolayer cell cultures (2–3-days old) into new plastic flasks. The 1/2 of cells from each flask was transferred into new one and incubated until confluency. The remaining cells were diluted in the medium and at the density of 2×10^5 distributed into 24-well plates (Nunc, Roskilde, Denmark) or into siliconized tubes, containing discs cut out from Spongostan^R (wet discs volume — 0.11 cm^3) and cells incubated for 48 hrs at 37°C. EC cultures in tubes with discs were incubated in gyratory water bath (100 rpm).

Induction of cytokine production

Two-dimensional BAECs cultures on flat plastic plates or three-dimensional cultures on Spongostan^R discs, after changing the medium to a new supplemented with 2% of FCS, were treated with cytokine inducers: Newcastle Disease Virus, Radom strain (NDV), 5 $TCID_{50}$/cell was used as interferon (IFN) inducer, LPS from *E. coli*, serotype 0111: B4 (Sigma) at a concentration of 10 μg/ml was used as TNF inducers. Cultures were additionally incubated at 37°C for 4 or 24 h, supernatants were collected, centrifuged and examined for IFN (24 h), TNF (4 h) activity.

Assay for cytokines

WEHI 164 — murine fibrosarcoma cells (ECACC No 8 702 251) were used to measure TNF activity by estimating the percentage of cytotoxicity according to the method of Allen et al. (2). Briefly, WEHI 164 cells were grown in a 96-well microtiter plate for 6 h, the medium was removed and replaced with 50 μl/well of a fresh one with actinomycin D (final concentration 0.5 μg/ml). 50 μl of serial 3-fold dilutions of the samples examined and standard recombinant rHuTNF-α (received from the Department of Bioorganic Chemistry, Center for Molecular and Macromolecular Research, Polish Academy of Sciences, Łódź) were added in triplicates to the wells and incubated for 24 h. The cytotoxic effect of TNF was determined by using MTT method

(18). The reciprocal of the highest dilution causing destruction of cells in 50% and compared to the standard was defined as 1 unit of TNF.

For IFN assay a cytopathic effect (CPE) inhibition method was used with vesicular stomatitis virus (VSV) as challenge. Briefly, IFN samples were serially diluted (0.3 log) in 96-microtiter plates in triplicates, and bovine embryonic fibroblasts (BF), a strain obtained by trypsynization of foetal skin, were added and allowed to form a confluent monolayer during 24 h of incubation at 37°C. VSV was added and the plates were incubated at 37°C until CPE in wells with VSV (virus control) was 100%. The reciprocals of sample dilution that protects 50% of cells against VSV were scored and calibrated in relation to a laboratory standard: human recombinant IFN-α88 (a generous gift of Erik Lundgren, Umea, Sweden) titrated against International Standard 69/19.

IFN activity was identified by neutralization with rabbit anti-Bov IFN-α_1 polyclonal antibodies (Genzyme). IFN containing samples were incubated for 3 h at room temperature with equal volumes of antibodies diluted 1 : 10, together with control samples to which the medium was added instead of antibodies, and titrated as described above. The inhibition of IFN activity by antibodies in comparison to control samples was expressed in percent.

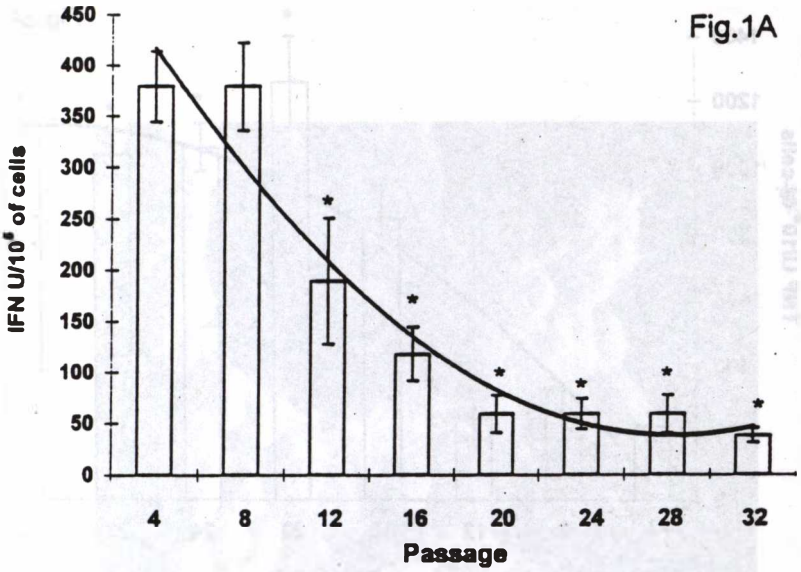
Statistics

Data were analysed using Student's t-test. Significance was reported as $p < 0.05$.

RESULTS AND DISCUSSION

The aim of this study was to assess the influence of culture conditions on the variations in cytokine production related to *in vitro* aging of BAECs cultures. BAECs were serially passaged in plastic flasks and cells at passages 4–32 were transferred into 24-well plastic plates (two-dimensional cultures) or cultivated on sponge discs cut out from Spongostan Special^R (three-dimensional cultures). As can be seen from Figure 1A, IFN production ability of BAECs decreased with the number of passages *in vitro,* while TNF production (Fig. 2A) increased with the number of passages *in vitro* till 20 passages and decreased slowly in higher passages. The changes in IFN and TNF production ability of BAECs were not caused by a significant difference in cell metabolism as glucose uptake per cell was maintained relatively constant 1 ng/cell/24h (data not shown) during all passages examined. IFN activity produced by BAECs in response to NDV was not neutralised by polyclonal rabbit anti-Bov IFN-α_1 antibodies, however, was sensitive for trypsin (0.25% trypsin for 1 h at 37°C) treatment, so we suppose that it was IFN-β type.

The changes in cytokine production ability of BAECs could be connected with two different mechanisms. Endothelial cells isolated from organism and cultivated *in vitro* on plastic plates or flasks, without contact with extracellular matrix (ECM), can exhibit several changes in phenotype and gene expression (7, 21). Moreover, *in vitro* cultivation is connected with multiplication of cells and their senescence, resembling processes which occur in aging organism. It was detected that morphological characteristics of endothelium isolated from



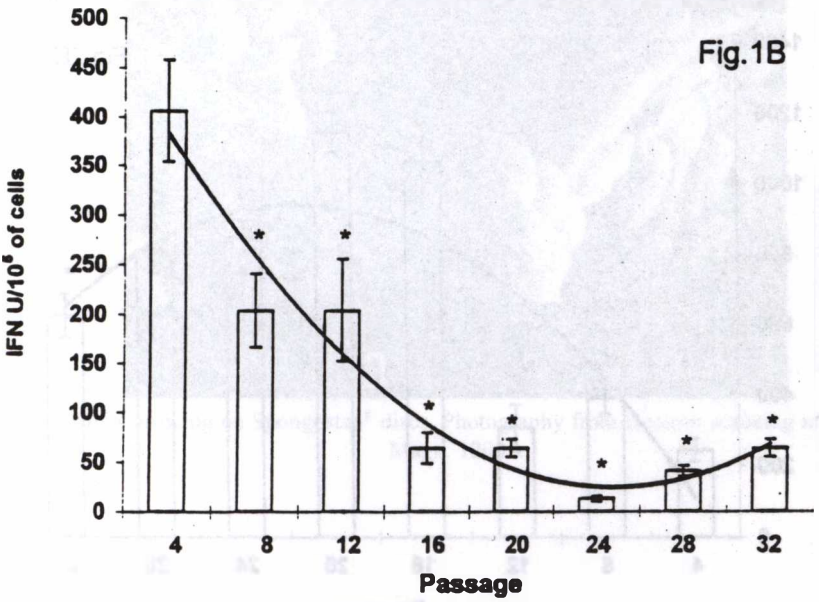
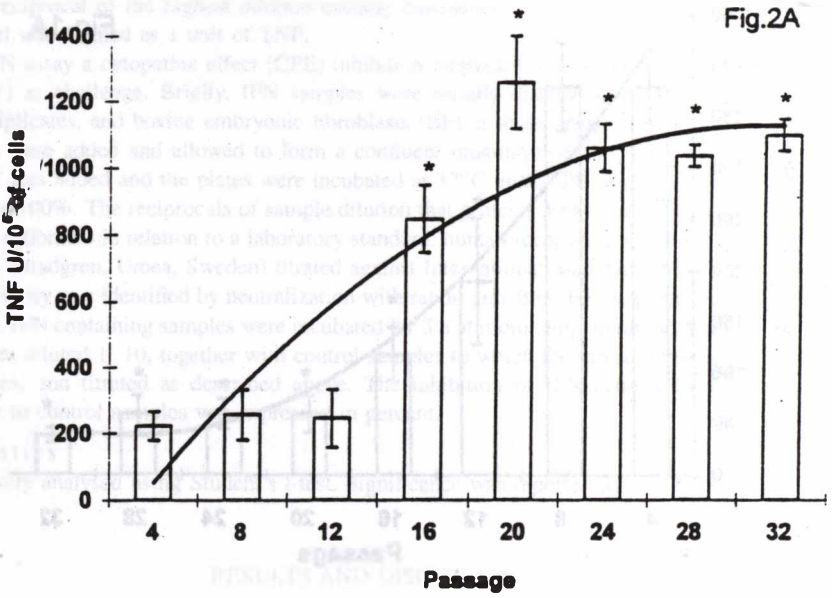


Fig. 1. IFN production by *in vitro* aging BAECs after induction with Newcastle disease virus. (A) two-dimensional cultures, (B) three-dimensional cultures; * statistically significant difference in comparison to IFN production in cells at 4-passage level



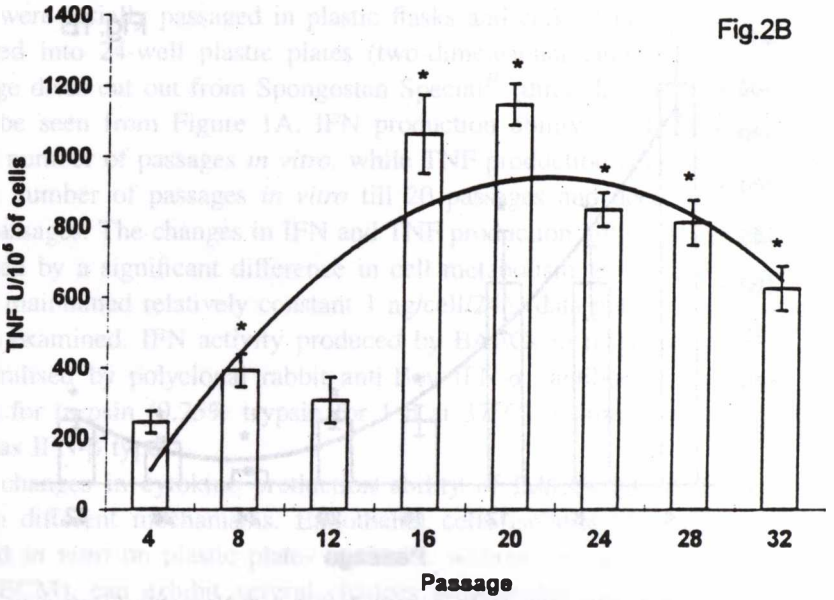


Fig. 2. TNF production by *in vitro* aging BAECs after induction with LPS. (A) two-dimensional cultures, (B) three-dimensional cultures; * statistically significant difference in comparison to IFN production in cells at 4-passage level

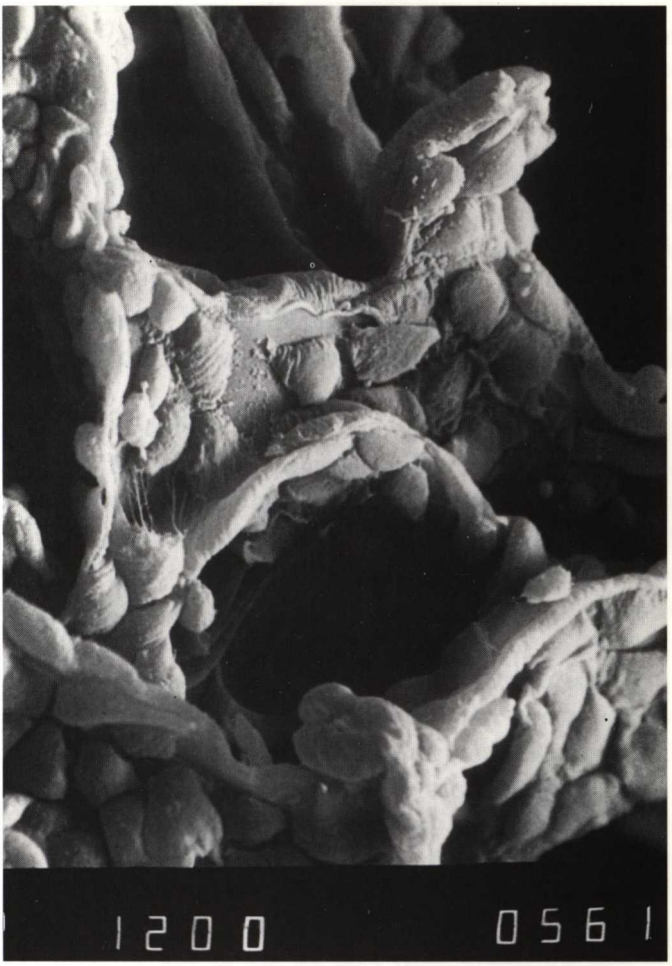


Fig. 3. BAECs growing on Spongostan[R] discs. Photography from electron scanning microscope. Magn. 1200 x

different human organs as brain, lung, renal glomeruli or bone marrow were gradually lost when cells were propagated in culture (3, 22). Changes in karyotype, in cellular levels of vonWillebrand factor and angiotensin converting enzyme (ACE), in levels of triglycerides and thiobarbituric acid reactive substances were also detected (5, 7). Moreover, enhancement of the expression of certain genes with concomitant decrease in another gene expression was also observed. For example, a significant increase in tissue inhibitor of metalloprotease 1 (TIMP1) was observed in mouse endothelium aged *in vitro,* with contemporary decrease in the expression of some metalloproteases, resulting in impaired migration of endothelial cells (20). Our data also strongly indicate that two cytokines production ability can be differentially regulated in endothelium aging *in vitro.* There are no papers concerning the influence of aging *in vitro* on cytokine production in bovine endothelium, however, a significant increase in the growth inhibitory effect of TNF on human endothelial cells aging *in vitro* was described (23).

On the other hand, conditions of *in vitro* cultures can significantly influence the phenotype of cells. For example, collagen coated surfaces have been shown to support better the growth of endothelial cells in comparison to plastic surfaces. Also the life-span of EC cultures can be prolonged in the presence of the fibroblast growth factor (FGF) (4, 21). In our study we tried to reverse the phenotypic changes, observed as decreased ability to produce IFN and increased ability to produce TNF, exhibiting by cultivated *in vitro* BAECs, by seeding BAECs at different passage level on three-dimensional scaffold — Spongostan^R. We suppose that this foam gelatine gel can mimic the extracellular matrix, present in the basement membrane of vessels. As yet several three-dimensional scaffolds have been examined, and some of them were found as useful for tissue engineering. Natural materials, as collagen have the potential advantage of specific cell interactions. Also synthetic sponge matrix fabricated from poly-L-lactic (PLLA) and polyglycolic acid (PGA) were widely used for tissue engineering (13). In our study BAECs seeded on Spongostan^R grown well on inner and outer surface of gelatine sponge (Fig. 3), but as can be seen from Figure 1B and Figure 2B, no significant differences in cytokine production ability of BAECs cultivated in three-dimensional and two-dimensional cultures were observed.

EC form a crucial part of the vascular component, regulating various physiological processes such as leukocyte adhesion, cytokine secretion and coagulation. EC cultivated *in vitro* are very often used in studies concerning their role in atherosclerosis, inflammatory and neoplastic disease processes. EC are the source of several inflammatory cytokines, chemokines and hematopoietic cytokines. However, when secretory activity of EC is examined *in vitro,* changes connected with their aging *in vitro* which cannot be reversed by three-dimensional

culture condition should be taken into consideration in final conclusions about the role of endothelial cells in the organism.

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