TISSUE ENGINEERING
Volume 11, Number 11/12, 2005
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Analysis of the Balance between Proliferation and Apoptosis of Cultured Vascular Smooth Muscle Cells for Tissue-Engineering Applications

P. ENGBERS-BUIJTENHUIJS, Ph.D.,[1,2] L. BUTTAFOCO, Ph.D.,[1] A.A. POOT, Ph.D.,[1] R.H. GEELKERKEN, M.D., Ph.D.,[2] J. FEIJEN, Ph.D.,[1] and I. VERMES, M.D., Ph.D.[1,2]

ABSTRACT

Tissue homeostasis, the balance between cell proliferation and apoptosis, is an important factor in tissue engineering. We describe a new method to analyze markers of both proliferation and apoptosis in a single assay to monitor growth behavior of cell cultures. Human vascular smooth muscle cells (VSMCs) were cultured either on gelatin-coated tissue culture polystyrene or in three-dimensional porous scaffolds composed of insoluble collagen and elastin. mRNA concentrations of cyclin E, as a marker of proliferation, and of tissue transglutaminase (tTG) as a marker of apoptosis, quantified by a real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and normalized to porphobilinogen deaminase mRNA concentrations, were analyzed. tTG mRNA expression levels were increased when apoptosis was induced by tumor necrosis factor-α in combination with cycloheximide or by culturing the cells in serum-free culture medium. Cyclin E mRNA expression levels were less altered in these cell cultures. Results were compared with several reference tests to measure apoptosis including DNA fragmentation, annexin V staining, and light microscopy. This RT-PCR method could be used to characterize cell growth behavior of VSMCs *in vitro*. In addition, it was shown that this test is suitable to measure the balance between proliferation and apoptosis of VSMCs present in tissue-engineered constructs.

INTRODUCTION

Cells for tissue-engineering applications are isolated from (human) tissue and cultured *in vitro*. There is significant variety between different batches of these cultures, which makes it difficult to perform experiments in a standardized way. In addition, it is difficult to evaluate the success of cell seeding and culturing in three-dimensional porous scaffolds without quantitative techniques to assess the growth behavior of these cells. The balance between proliferation and apoptosis of vascular cells is responsible for mediating profound changes in vascular architecture in normal development and disease[1] and therefore important for the success of tissue engineering. Events of both proliferation and apoptosis can be measured *in vitro* by several qualitative as well as quantitative techniques. Most of the quantitative methods used to study apoptosis in cell populations are based on flow cytometry of cells in suspension.[2–4] Flow cytometry can also be performed with adherent cells after detachment of the cells from their support. However, cell detachment induces apoptosis by itself.[5] Therefore,

[1]Polymer Chemistry and Biomaterials Group, Faculty of Science and Technology, University of Twente, Enschede, The Netherlands.
[2]Department of Clinical Chemistry and Department of Vascular Surgery, Medisch Spectrum Twente Hospital Group, Enschede, The Netherlands.

flow cytometry is not an optimal quantitative technique to measure apoptosis in adherent cells.[6]

A new approach is to analyze mRNA expression levels of an apoptotic marker. Tissue transglutaminase (tTG) is a multifunctional transamidating acyltransferase that becomes activated during the latter phase of apoptosis and that plays a role in the formation of apoptotic bodies. Activated tTG induces irreversible cross-links in and between cytoplasmic proteins to produce a large, stable, insoluble protein scaffold.[7] This cross-linking of proteins stabilizes cell and membrane structures of disintegrated cells and apoptotic bodies.[8] Volokhina *et al.*[9] showed that tTG mRNA expression could be used as a trace marker for detection and quantification of apoptosis of both circulating and adherent cells. mRNA expression levels were measured by semiquantitative real-time reverse transcriptase polymerase chain reaction (RT-PCR).[10] In the present study, we describe the optimization of this RT-PCR technique to determine tTG mRNA expression levels for analysis of apoptosis in vascular smooth muscle cell (VSMC) cultures. A DNA fragmentation assay measuring the incorporation of propidium iodide (PI) by flow cytometry,[5,11] light microscopy, and a time-resolved fluorometric assay using europium-labeled annexin V[12] were used as reference tests to measure apoptosis.

Because markers of both apoptosis and proliferation must be taken into account when considering growth behavior,[1] we measured mRNA expression levels of cyclin E as a marker of proliferation. Cyclin E regulates the transition from the G_1 to the S phase of the cell cycle. A high level of cyclin E protein facilitates this transition, indicating enhanced proliferation.[13,14] In this way, events of both proliferation and apoptosis can be analyzed in a single assay. An additional advantage of this method is that cells do not have to be detached from the support because RNA can be directly isolated from adherent cells. Therefore, this method is ideal for tissue-engineering applications because RNA can be isolated from cells cultured inside porous scaffolds. Effects of different types of porous structures on the growth behavior of cells cultured inside the scaffolds can be analyzed in a quantitative way.

In the present article, culturing of VSMCs on gelatin-coated tissue culture polystyrene (g-TCPS) was used as a model to develop the methodology to analyze the balance between proliferation and apoptosis *in vitro*. To verify whether this method could be used for tissue-engineering purposes, VSMCs were seeded and cultured in three-dimensional porous scaffolds composed of insoluble collagen and elastin for tissue engineering of small-diameter blood vessels, as previously described.[15] By analyzing the tTG and cyclin E mRNA expression levels of these cells, the influence of two scaffold cross-linking procedures on VSMC growth behavior was investigated.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO-BRL (Breda, The Netherlands). Penicillin, streptomycin, fetal bovine serum, and trypsin–ethylenediaminetetraacetic acid (EDTA) were purchased from Biowhittaker (Verviers, Belgium). Gelatin type B from bovine skin, tumor necrosis factor-α (TNF-α), cycloheximide (CHX), bovine serum albumin (BSA), propidium iodide (PI), DNase-free RNase, 2-mercaptoethanol, *N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), and poly(propylene glycol)-bis-(2-aminopropyl ether) (J230) were obtained from Sigma-Aldrich (St. Louis, MO). Mouse monoclonal antibodies (mAbs) against human α-smooth muscle actin (α-SMA) and human vimentin and fluorescein isothiocyanate-labeled rabbit anti-mouse immunoglobulins (RAM–FITC) were purchased from DakoCytomation (Glostrup, Denmark). Human serum was acquired by overnight coagulation of blood (collected from healthy volunteers), subsequently pooled, and stored at -80°C. A QIAmp RNA blood mini kit was from Qiagen (Hilden, Germany). Primers, dithiothreitol (DTT), and Moloney murine leukemia virus (M-MLV) reverse transcriptase were obtained from Invitrogen (Paisley, UK). dNTPs and enhancement solution were from Amersham Biosciences/GE Healthcare (Cambridge, UK). RNase inhibitor was from Roche (Basel, Switzerland). *N*-(2-Hydroxyethyl) piperazine-*N'*-(2-ethanesulfonic acid) (HEPES) was from Brunschwig (Amsterdam, The Netherlands). Other buffer components and salts were obtained from Merck (Darmstadt, Germany). Insoluble collagen (type I from bovine Achilles tendons) and insoluble elastin (from equine ligamentum nuchae, purification as in Daamen *et al.*[16]) were kindly donated by T.H. van Kuppevelt (Department of Biochemistry, Katholieke Universiteit Nijmegen, Nijmegen, The Netherlands). Annexin V was kindly donated by C. Reutelingsperger (University of Maastricht, Maastricht, The Netherlands).

Methods

Isolation and culturing of VSMCs on g-TCPS. VSMCs were isolated from human umbilical veins according to the method of Heimli *et al.*[17] with some minor modifications as previously described.[15] Cells were cultured on gelatin-coated (0.5%, w/v) tissue culture polystyrene (g-TCPS), using DMEM containing 10% (v/v) heat-inactivated (30 min, 56°C) pooled human serum, 10% (v/v) heat-inactivated (30 min, 56°C) fetal bovine serum, penicillin (50 U/mL), and streptomycin (50 μg/mL) (standard culture medium).[17] Culture medium was refreshed every

2–3 days. Cells were cultured in an incubator at 37°C in a humidified atmosphere containing 5% CO_2.[18] When subconfluent cultures were obtained, cells were detached from the support with 0.125% (w/v) trypsin–0.05% (w/v) EDTA and subcultured for several passages (maximum, 15; split ratio, 1:3).[19] To analyze apoptosis and/or proliferation, VSMCs were cultured to 80% of confluence (time point 0) and subsequently cultured in standard culture medium (control cells) or in serum-free culture medium to induce apoptosis in a natural (mild) way.[20] To chemically induce apoptosis, cells were treated with tumor necrosis factor-α (TNF-α) in combination with cycloheximide (CHX) (3 nM and 50 μM in standard culture medium, respectively).

Identification of VSMCs. VSMCs were identified with mAbs against human α-SMA and human vimentin.[17,18] Fluorescence of the secondary antibody, RAM–FITC, was examined by immunofluorescence microscopy and by flow cytometry as described previously.[15] Negative controls were obtained by omitting primary antibodies and by staining human umbilical vein endothelial cells (HUVECs) and human dermal fibroblasts with the antibodies used.

DNA fragmentation assay. DNA fragmentation in cell cultures on g-TCPS was determined by flow cytometry, using PI according to Nicoletti *et al*.[11] After several culture time intervals, samples of cell culture medium were collected and adherent cells were detached from their support, using 0.125% trypsin–0.05% EDTA solution. Detached cells and cells present in the culture medium were washed and fixed in 70% ethanol. After another wash, cells were stained with 15 μM PI for 30 min at 37°C[2] and the PI fluorescence of individual cells was measured with a Coulter EPICS XL flow cytometer (Beckman Coulter, Fullerton, CA), using SYSTEM II software with the XL-2 or DOS configuration. Excitation was elicited at 488 nm with an argon laser and measured with long-pass ($>$570 nm) filters. For each sample 10,000 events were measured and data were analyzed with the Coulter program EXPO II.

Europium-labeled annexin V time-resolved fluorometric assay. For the time-resolved fluorometric assay, three fractions of adherent cell cultures grown on g-TCPS were prepared and analyzed as previously described.[12] Fraction 1 consisted of adherent cells; these cells were analyzed while growing on their support (without detachment by trypsinization). Supernatants of the adherent cell cultures were collected and two other fractions were isolated: floating cells and apoptotic bodies. After centrifugation of the supernatants (low speed [1000 $\times$ *g*], 3 min, room temperature), apoptotic cells detached from their support were obtained from the pellets (fraction 2, floating cells). Supernatants of these centrifuged samples were collected and centrifuged (high speed [3500 $\times$ *g*], 15 min, room temperature). Apoptotic bodies derived from the apoptotic cells as a result of a final stage of apoptosis were then obtained from the pellets (fraction 3). To analyze apoptosis, the three fractions of the cell cultures (adherent cells, floating cells, and apoptotic bodies) were separately washed with a solution of 10 mM HEPES, supplemented with 137 mM NaCl, 2.68 mM KCl, 1.7 mM $MgCl_2$, 25 mM glucose, and 2.5 mM $CaCl_2 \cdot 2H_2O$ at pH 7.4 (HEPES buffer). Adherent cells (fraction 1) were then incubated with europium-labeled annexin V (final concentration, 0.4 mg/L in HEPES buffer) for 30 min at room temperature. Pellets of floating cells (fraction 2) and apoptotic bodies (fraction 3) were resuspended in europium-labeled annexin V (final concentration, 0.4 mg/L) in HEPES buffer for 30 min at room temperature. After washing with HEPES buffer, the three fractions were incubated with commercially available enhancement solution[21] for 5 min at room temperature to convert the europium label into the highly fluorescent chelate. Time-resolved fluorescence was measured in each sample on a Wallac VICTOR fluorescence analyzer (PerkinElmer Life Sciences, Turku, Finland). Excitation and emission wavelengths were 340 and 615 nm, respectively. Fluorescence was normalized relative to control cell cultures. By analyzing apoptosis in three different cell fractions of the adherent cell cultures, the occurrence of apoptosis/anoikis could be measured without manipulation of the cell cultures.[12]

Light microscopy. After cells were cultured to 80% confluence, apoptosis/anoikis was induced by TNF-α/CHX or by culturing the cells in serum-free culture medium. Adherent cells still present on their culturing support and apoptotic cells detached from their support (floating cells) were analyzed. An aliquot of culture medium containing floating cells was centrifuged on glass slides at 700 rpm for 10 min with low acceleration, using a Shandon Cytospin Cytocentrifuge (Thermo Electron, Waltham, MA). Adherent cells still present on their culturing support and floating cells collected on the glass slides were fixed with methanol and stained with May–Grünwald–Giemsa, and their appearance was observed by normal light microscopy.

CyQUANT cell proliferation assay. Numbers of VSMCs were quantified by CyQUANT cell proliferation assay according to the manufacturer's instructions (Molecular Probes, Leiden, The Netherlands). Cells were rinsed with phosphate-buffered saline (PBS), detached from their support with 0.125% (w/v) trypsin–0.05% (w/v) EDTA, and stored at -80°C. Various dilutions were prepared with cell lysis buffer (Molecular Probes) supplemented with 180 mM NaCl, 1 mM EDTA, and

DNase-free RNase (1.35 Kunitz units/mL). Samples were then incubated for 1 h at room temperature to remove the RNA and single-stranded DNA. Finally, samples were mixed with CyQUANT dye and, after 2 min, fluorescence was measured in 96-well plates with a Wallac VICTOR fluorescence analyzer (PerkinElmer Life Sciences). Excitation and emission wavelengths were 480 and 520 nm, respectively. The measured fluorescence intensities were correlated to the amount of VSMCs by comparison with a calibration curve made by means of dilutions with known concentrations of VSMCs from a subconfluent culture on g-TCPS. Cells were detached from the support with 0.125% (w/v) trypsin–0.05% (w/v) EDTA, counted with a Bürker hemacytometer, and then analyzed in the same way as described above to obtain a calibration curve.[22]

RNA isolation and real-time RT-PCR method. RNA isolation was performed with a QIAamp RNA blood mini kit (Qiagen) according to the manufacturer's instructions. Adherent VSMCs were washed with PBS and total RNA content was harvested by disrupting the cell membranes with 600 μL of cell lysis buffer (buffer RLT; Qiagen) containing 1% (v/v) 2-mercaptoethanol, using a mechanical homogenizer. This procedure did not include trypsinization of the adherent VSMCs. Standard cDNA synthesis was performed as described by Volokhina *et al.*[9] and mRNA expression levels for tTG and cyclin E were determined by a TaqMan-based, real-time RT-PCR technique according to Heid *et al.*[10] Fragments of the tTG sequence were amplified with forward primer 5′-GCCACTTCATTTTGCTCTTCAA-3′ and reverse primer 5′-TCCTCTTCCGAGTCCAGGTACA-3′ at a final concentration of 300 nM and probe sequence 5′-CCTGGTGCCCAGCGGATGCT-3′ at a final concentration of 200 nM.[9] Fragments of the cyclin E sequence were amplified with forward primer 5′-CTCCAGGAAGAGGAAGGCAA-3′ and reverse primer 5′-TGAAGAAATGGCCAAAATCGA-3′ at a final concentration of 300 nM and probe sequence 5′-TTTTTGCAGGATCC-3′ at a final concentration of 200 nM.[14] Probes were labeled at the 5′ end with reporter fluorophore FAM (carboxyfluorescein) and at the 3′ end with quencher fluorophore TAMRA (tetramethylrhodamine), which served as a quencher. Primer and probe concentrations were optimized according to the guidelines of the provider. mRNA expression levels of the two proteins were normalized to mRNA expression levels of porphobilinogen deaminase (PBGD) as described by Volokhina *et al.*[9] To investigate whether mRNA expression levels of PBGD could be used to normalize the cyclin E and tTG mRNA expression levels of VSMCs, the influence of serum starvation and of TNF-α/CHX treatment on the PBGD mRNA expression of VSMCs was checked. After total RNA was isolated from VSMCs cultured in standard culture medium, serum-free culture medium, or TNF-α/CHX-containing standard culture medium, the amount of RNA was determined by measuring the absorbance at 260 nm (A_{260}), using a spectrophotometer. The purity of the isolated RNA was analyzed by using the spectrophotometer to measure the A_{260}/A_{280} ratio. PBGD mRNA expression levels in 1 μg of RNA were then analyzed. The number of cycles that it takes for the amplification plot to reach the threshold limit is called the threshold cycle (C_t value); this was used for quantification. mRNA expression levels of tTG and cyclin E in cells cultured on g-TCPS were compared with levels at time point 0. mRNA expression levels in cells cultured on cross-linked scaffolds were compared with levels in cells cultured on non-cross-linked (native) scaffolds.

Culturing VSMCs in three-dimensional porous scaffolds composed of collagen and elastin. Three-dimensional (circular) porous scaffolds for tissue-engineering applications (diameter, 16 mm; thickness, 2 mm; porosity, 98%; pore size, about 200 μm[15]) were used to examine the interaction of VSMCs with the scaffolds. Scaffolds were composed of insoluble type I collagen (derived from bovine Achilles tendons) and insoluble elastin (from equine ligamentum nuchae), prepared by freeze-drying and optimized in terms of pore size and cross-link density.[15] Cross-linking of the scaffolds was performed either with a water-soluble carbodiimide in combination with a succinimide (EDC–NHS) or with a diamine (J230) in the presence of EDC–NHS, to improve the physical and mechanical properties.[23] Scaffolds were sterilized with 70% ethanol and washed three times with PBS before VSMC seeding. VSMCs were seeded in the scaffolds at a density of 200,000 cells/cm^2, using filtration seeding as described by Li *et al.*[24], and subsequently cultured in 24-well plates under static conditions for 14 days in DMEM culture medium. Culture medium was refreshed every 2–3 days. Cell attachment and growth were analyzed by histology, using a standard elastic von Gieson staining procedure on transverse sections of the tissue-engineered scaffolds (Laboratory of Pathology Oost-Nederland, Enschede, The Netherlands).

Statistical analysis. Data represent means $\pm$ standard error of the mean (SEM) of 5 to 10 experiments performed in duplicate. Statistical analyses of the influences of serum-free medium and TNF-α/CHX compared with control medium on VSMC cultures were performed by Wilcoxon rank sum test with correction for multiple testing (Holms testing). Statistical analyses of DNA fragmentation and tTG mRNA expression levels for up to 240 h were performed by analysis of variance (ANOVA) and a post hoc Tukey's honestly significant difference (HSD) test. In both cases $p < 0.05$ was considered statistical significant.

RESULTS

Identification of VSMCs

g-TCPS-cultured VSMCs, isolated from human umbilical veins, showed a characteristic morphology consisting of a hill-and-valley pattern 5 days after reaching confluence. Filaments of α-SMA (in 60% confluent cultures) were visualized by immunofluorescence microscopy. Human skin fibroblasts and HUVECs showed neither this characteristic hill-and-valley pattern nor this pattern of fluorescence (data not shown). Quantitative analyses of the presence of α-SMA filaments and vimentin were done by flow cytometry. Using the mAb against human α-SMA, 98% of the treated cells were positively stained, relative to 2% of the negative control, from which the first antibody was omitted. With the mAb against human vimentin, 81% of treated cells were positively stained, relative to 2% of the negative control cells, indicating that cultured cells are of mesenchymal origin.[18] Human skin fibroblasts and HUVECs were not positively stained with the mAb against α-SMA (data not shown).

DNA fragmentation assay

By means of flow cytometry of VSMCs cultured on g-TCPS, diagrams of forward scatter (FSC) against side scatter (SSC) were obtained. A change in scatter diagram in the lower scatter regions could be observed when cells were cultured in serum-free culture medium or when cells were incubated with TNF-α/CHX compared with control cells. Histograms of PI fluorescence of human VSMCs showed three distinct peaks, indicating three separate cell populations: a G_0/G_1 region of normal diploid cells, a G_2/M region of dividing cells with a double amount of DNA, and an A_0 region of hypodiploid cells.[2] The subpopulation of A_0 cells showed reduced DNA stainability, indicating apoptotic cells in which DNA fragments appear and cell fragmentation has occurred.[2,3] Percentages of apoptotic cells compared with the total amount of cells, and compared with percentages of apoptotic cells at time point 0, were calculated. Percentages of apoptotic cells were increasing over time for TNF-α/CHX-treated cell cultures, whereas no increase over time of percentages of apoptotic cells was observed for VSMCs cultured in standard culture medium with and without serum for up to 48 h, as can be seen in Fig. 1.

DNA fragmentation assay analysis of apoptosis in control cell cultures for up to 240 h showed an increase in A_0 cells (Fig. 2A).

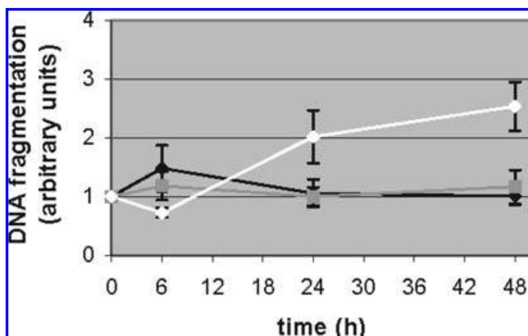

FIG. 1. Apoptotic cell death of VSMCs cultured on g-TCPS, measured by a DNA fragmentation assay as a function of time. Cells were cultured in serum-containing culture medium (control cells, solid diamonds) or in serum-free culture medium (shaded squares) or were treated with 3 nM TNF-α and 50 μM CHX in serum-containing culture medium (open diamonds) for various amounts of time. DNA was stained with PI and fluorescence was analyzed by flow cytometry. Percentages of apoptotic cells compared with the total amount of cells and with percentages of apoptotic cells at time point 0, were calculated and plotted against time.

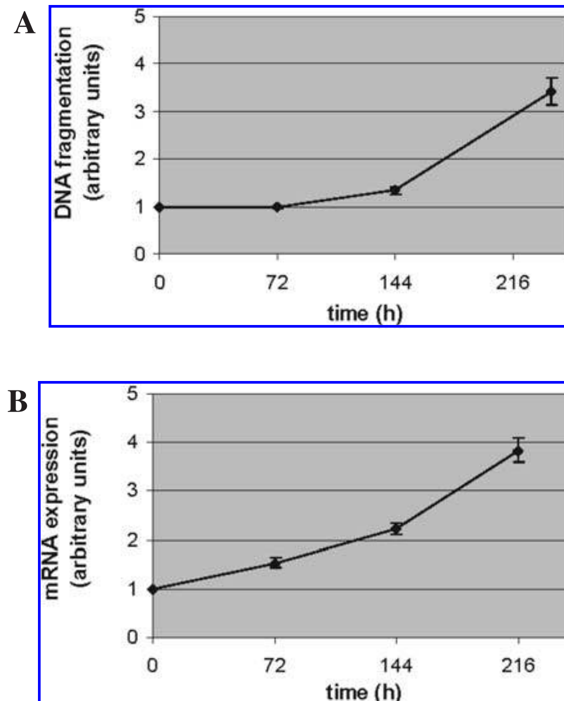

FIG. 2. Apoptosis of VSMCs cultured on g-TCPS, measured by analyzing DNA fragmentation and tTG mRNA expression levels as a function of time. Cells were cultured in serum-containing culture medium for various amounts of time. DNA was stained with PI and fluorescence was analyzed by flow cytometry. Percentages of apoptotic cells, compared with the total amount of cells measured and compared with percentages of apoptotic cells at time point 0, were calculated and plotted against time (**A**). tTG mRNA expression levels were determined by a semiquantitative real-time RT-PCR method. tTG mRNA expression levels, compared with levels at time point 0 and normalized to mRNA expression levels of PBGD, were calculated and plotted against time (**B**).

Light microscopy

Light microscopy revealed that treatment of VSMC cultures with TNF-α and CHX, and culturing the cells in serum-free culture medium, both induce detachment of adherent VSMCs (anoikis). After 24 h, control cell cultures had grown from 80% to approximately 90% confluency (Fig. 3a), whereas after culturing the cells for 24 h in serum-free culture medium, approximately 70% confluency was observed (Fig. 3b). TNF-α/CHX-treated cell cultures showed a confluency of approximately 40% after 24 h (Fig. 3c). No morphological abnormalities were observed in the attached cells. After treatment of the VSMC cultures with TNF-α and CHX, or after culturing the cells in serum-free culture medium, cells detached from their culturing support were found in the culture medium (floating cells). These floating cells showed cell blebbing and apoptotic body formation (Fig. 3d).

Europium-labeled annexin V time-resolved fluorometric assay

The europium-labeled annexin V time-resolved fluorometric assay was performed to measure apoptosis in cell cultures after 24 h of TNF-α/CHX treatment compared with control cultures. A significant increase (2.7 $\pm$ 0.04) in time-resolved fluorescence was found. In addition, after culturing the cells in culture medium without serum, a slight but significant increase (1.3 $\pm$ 0.03) in time-resolved fluorescence was found compared with control cultures, indicating increased apoptosis in the adherent VSMCs.

tTG mRNA expression of VSMCs cultured on g-TCPS

At least 25 μg of total RNA was isolated from cell cultures as determined by spectrophotometry. The A_{260}/A_{280} ratio was between 1.7 and 1.9, indicating pure RNA. PBGD mRNA expression levels in cultured VSMCs were not influenced by serum starvation or TNF-α/CHX treatment. This allowed us to use PBGD mRNA expression levels to normalize tTG and cyclin E mRNA expression levels for quantification. Analysis of tTG mRNA expression levels of control cell cultures on g-TCPS for up to 216 h showed an increase in tTG mRNA expression, using the semiquantitative real-time RT-PCR method (Fig. 2B). This increase resembles the increase in A_0 cells measured by the DNA fragmentation assay (Fig. 2A). Results of analyses of tTG mRNA expression levels over time of VSMCs cultured on g-TCPS in culture medium with or without serum or treated with TNF-α/CHX in standard culture medium are shown in Fig. 4A. After 24 h, an increase in tTG mRNA expression level was observed for cells cultured in culture medium without serum and for cells treated with TNF-α/CHX, whereas no increase was observed for cells cultured in standard culture medium. tTG mRNA expression levels of TNF-α/CHX-treated cell cultures corresponded with results of the DNA fragmentation assay (Fig. 1), the europium-labeled annexin V time-resolved fluorometric assay, and light mi-

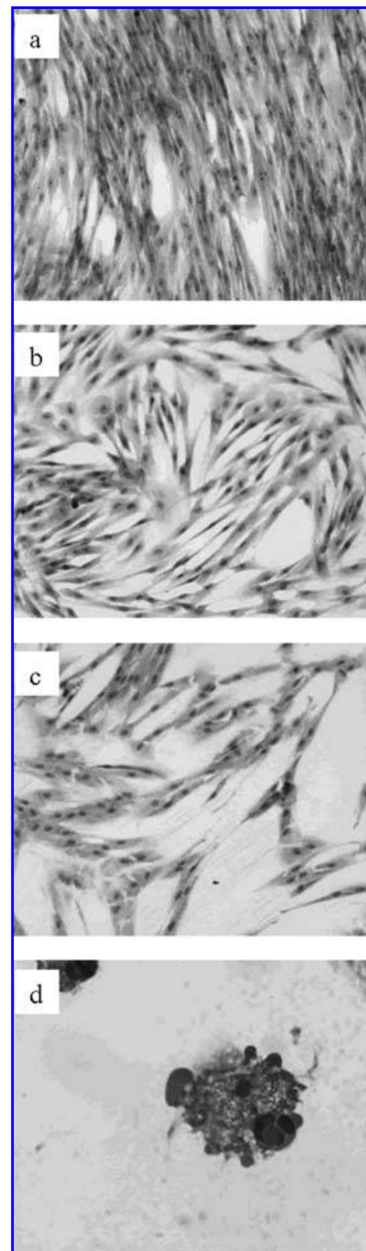

FIG. 3. Morphology of VSMC cultures. Cells were cultured to 80% confluence on gelatin-coated culture flasks and cultured for 24 h in standard culture medium with (**a**) or without (**b**) serum, or were treated with 3 nM TNF-α and 50 μM CHX in serum-containing culture medium (**c**). Adherent cells still present on the surface of gelatin-coated culture flasks were stained according to the May–Grünwald–Giemsa staining procedure (**a**–**c**). Apoptotic VSMCs that had detached from their support as a result of an early stage of apoptosis (floating cells) were centrifuged onto glass slides and stained according to the May–Grünwald–Giemsa staining procedure (**d**). Original magnification, $\times$100.

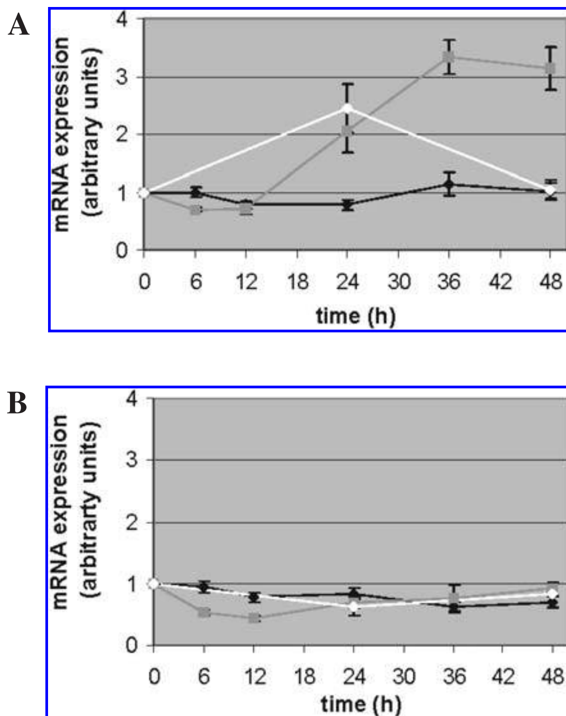

FIG. 4. Apoptosis and proliferation of VSMCs cultured on g-TCPS, measured by analyzing tTG (**A**) and cyclin E (**B**) mRNA expression levels as a function of time. Cells were cultured in serum-containing culture medium (control cells, solid diamonds) or in serum-free culture medium (shaded squares) or were treated with 3 nM TNF-α and 50 μM CHX in serum-containing culture medium (open diamonds) for various periods of time. mRNA expression levels were determined by a semiquantitative RT-PCR method. tTG mRNA expression levels compared with levels at time point 0 and normalized to mRNA expression levels of PBGD, were calculated and plotted against time.

croscopy (Fig. 3). tTG mRNA expression levels of cells cultured in serum-free culture medium corresponded with results of the europium-labeled annexin V time-resolved fluorometric assay and light microscopy (Fig. 3). After culturing the cells for 48 h in serum-free culture medium, DNA fragmentation was still enhanced (Fig. 1), but tTG mRNA expression levels were not enhanced (Fig. 4A), compared with control cell cultures.

Cyclin E mRNA expression of VSMCs cultured on g-TCPS

Cyclin E mRNA expression levels as a function of time in VSMCs cultured on g-TCPS in culture medium with or without serum and in serum-containing culture medium supplemented with TNF-α and CHX are shown in Fig. 4B. After 6 and 12 h, the cyclin E mRNA expression level in cells cultured in serum-free culture medium was reduced compared with that in cells cultured in standard culture medium. After more than 24 h, no differences were observed. No significant differences in cyclin E mRNA expression levels were observed in cells cultured in standard culture medium supplemented with TNF-α and CHX. These data were confirmed by data obtained with the CyQUANT cell proliferation assay.

Tissue-engineering application

VSMCs adhered to and grew in multilayers on top of and inside three-dimensional porous structures composed of collagen and elastin, as observed by histology.[15] mRNA could be isolated from the tissue-engineered scaffolds. The expression levels of PBGD in these cultured VSMCs were not influenced by scaffold properties. This allowed us to use PBGD mRNA expression levels to normalize tTG and cyclin E mRNA expression levels for quantification. No significant changes were found in either cyclin E or tTG mRNA expression levels of VSMCs cultured in porous scaffolds, cross-linked either with EDC–NHS or with J230 in the presence of EDC–NHS, compared with native scaffolds, as can be seen in Fig. 5.

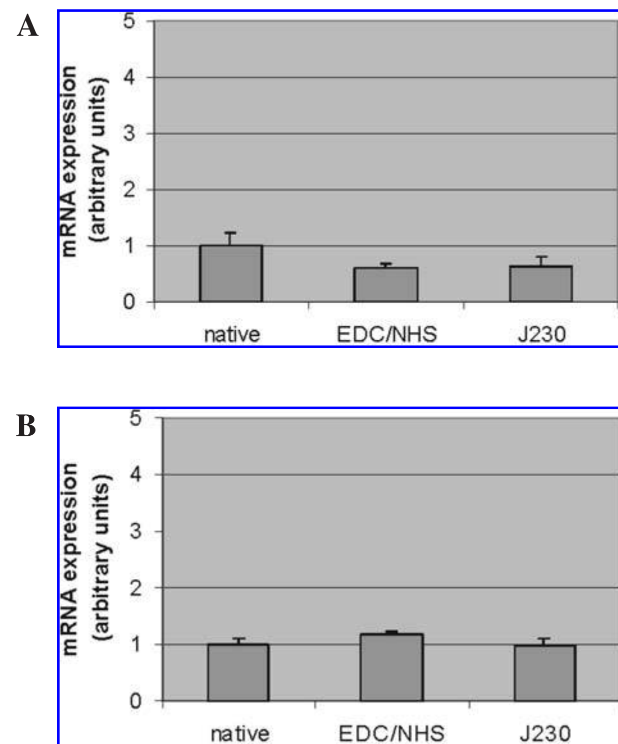

FIG. 5. Apoptosis (**A**) and proliferation (**B**) of VSMCs cultured for 14 days on tissue-engineered scaffolds were determined by analyzing tTG and cyclin E mRNA expression levels, respectively. Scaffolds were composed of collagen and elastin and cross-linked with either a carbodiimide in combination with a succinimide (EDC–NHS) or with a diamine (J230) in the presence of EDC–NHS. mRNA expression levels of tTG and cyclin E were determined by a semiquantitative real-time RT-PCR method and mRNA expression levels of cells cultured on cross-linked scaffolds were compared with mRNA expression levels of cells cultured on native (non-cross-linked) scaffolds.

DISCUSSION

A pure VSMC culture of mesenchymal origin[18] was used in this study to analyze the balance between proliferation and apoptosis. Cultured VSMCs on g-TCPS grew in overlapping layers in a series of hills and valleys as described in the literature.[17] The appearance of this organization at confluence and the presence of α-smooth muscle actin filaments distinguish these cultured cells from endothelial cells, fibroblasts, and myofibroblasts.[18,19] From the results of our reference DNA fragmentation assay using flow cytometry and the europium-labeled annexin V time-resolved fluorometric assay to measure apoptosis, it can be concluded that incubation with TNF-α and CHX induces apoptosis in VSMCs cultured on g-TCPS in a time-dependent manner.[25] These results were confirmed by light microscopy. In addition, from the results of the europium-labeled annexin V time-resolved fluorometric assay and light microscopy, it was shown that culturing VSMCs in serum-free culture medium also induces apoptosis. This is in agreement with other research that shows an apoptosis-inducing effect on culturing VSMCs in serum-free culture medium.[20,26] However, DNA fragmentation was not observed when VSMCs were cultured in serum-free culture medium. We suggest that, because of the elevated tTG protein level, stable apoptotic bodies are formed by stabilization of cell and membrane structures and that these apoptotic bodies are washed away during the procedure for flow cytometric analyses.[12] Using the semiquantitative real-time RT-PCR method described above, enhanced tTG mRNA expression was shown in VSMCs cultured on g-TCPS under several apoptosis-inducing conditions. When VSMCs cultured on g-TCPS in standard culture medium reach confluence (after 4–5 days) and the cells are treated with TNF-α and CHX for 24 h, enhanced DNA fragmentation, detachment of adherent VSMCs from the surface, annexin V exposure on the outer surface of the plasma membrane, and enhanced tTG mRNA expression occur, indicating that apoptosis is induced and that the methods are correlated. After 24 h of TNF-α/CHX treatment the tTG mRNA expression level of VSMCs is highly enhanced, but after 48 h the tTG mRNA expression level falls back to the control level. This is in contrast with DNA fragmentation, which is highly enhanced after 24 and 48 h of TNF-α/CHX treatment. This discrepancy is due to the different specificities of the two assays used. We have shown previously that in contrast to DNA fragmentation, tTG mRNA expression levels can distinguish between apoptotic and necrotic cell death.[9] Accordingly, our observations indicate that after 48 h of TNF-α/CHX treatment, cells are already in the late apoptotic/necrotic stage of the apoptotic cascade.

When growth behavior is taken into consideration, neither apoptosis nor proliferation alone will give satisfactory information; therefore markers of both biological events should be measured. With the described semiquantitative real-time RT-PCR method it is possible to measure both events in a single assay by analyzing cyclin E and tTG mRNA expression levels in VSMCs.[9,13,14,27,28] PBGD mRNA expression levels can be used for this purpose to normalize both tTG and cyclin E mRNA expression levels to correct for differences in total RNA content. Cyclin E mRNA expression levels were not significantly altered in VSMCs cultured on g-TCPS in standard culture medium for up to 48 h. VSMCs cultured in serum-free culture medium showed reduced cyclin E mRNA expression levels after 6 and 12 h compared with cells cultured in serum-containing medium, possibly because of the lack of growth factors present in normal serum. But after 24 and 36 h we did not find significant differences in cyclin E mRNA expression levels between the two cultures, suggesting an adaptation of the cell cycle.[29] In addition, TNF-α/CHX treatment did not influence cyclin E mRNA expression of the cells. Accurate and reproducible analysis of both proliferation and apoptosis of VSMCs within a single test is now possible, using the described semiquantitative real-time RT-PCR method.

We showed that the method is suitable for tissue-engineering applications by analyzing the cyclin E and tTG mRNA expression levels of VSMCs cultured in three-dimensional porous structures composed of collagen and elastin. These scaffolds are under investigation for tissue engineering of small-diameter blood vessels.[15] Cross-linking of the scaffolds either with a carbodiimide (EDC–NHS) or with a diamine (J230) in the presence of the carbodiimide does not influence VSMC growth behavior as determined by analyzing the balance between apoptosis and proliferation, using the RT-PCR method described in this article. These quantitative findings confirm our qualitative observations using histology as previously described.[15] We suggest that this method can be used to characterize and compare the cell growth behavior of different batches of cells. In addition, other mRNA expression levels of the same samples can easily be analyzed in a similar way, such as the mRNA expression of extracellular matrix proteins of VSMCs as described by Seliktar *et al*.[30] With this approach it will be possible to culture cells in a standardized way, not only to obtain a tissue-engineered blood vessel but also for other tissue-engineering purposes using a variety of cell types.

ACKNOWLEDGMENTS

W. Daamen and A. van Kuppevelt (Radboud Universiteit Nijmegen, Nijmegen, The Netherlands) are acknowledged for the donation of collagen and elastin. R.A.I. de Vos and L.M.T. Sterk (Laboratory of Pathology Oost-Nederland, Enschede, The Netherlands) are acknowledged for performing histology. Dr. C. Reutelingsperger (University of Maastricht, The Netherlands)

is acknowledged for the donation of annexin V. This project was financially supported, in part, by IOP Senter (Den Haag, The Netherlands) (Project IIE00003).

REFERENCES

1. Vermeulen, K., Berneman, Z.N., and Van Bockstaele, D.R. Cell cycle and apoptosis. Cell Prolif. **36,** 165, 2003.
2. Vermes, I., Haanen, C., and Reutelingsperger, C. Flow cytometry of apoptotic cell death. J. Immunol. Methods **243,** 167, 2000.
3. Darzynkiewicz, Z., Bedner, E., and Smolewski, P. Flow cytometry in analysis of cell cycle and apoptosis. Semin. Hematol. **38,** 179, 2001.
4. Michie, J., Akudugu, J., Binder, A., Van Rensburg, C.E.J., and Bohm, L. Flow cytometric evaluation of apoptosis and cell viability as a criterion of anti-tumour drug toxicity. Anticancer Res. **23,** 2675, 2003.
5. Micoud, F., Mandrand, B., and Malcus-Vocanson, C. Comparison of several techniques for the detection of apoptotic astrocytes *in vitro*. Cell Prolif. **34,** 99, 2001.
6. van Engeland, M., Ramakers, F.C.S., Schutte, B., and Reutelingsperger, C.P.M. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry **24,** 131, 1996.
7. Fesus, L., and Piacentini, M. Transglutaminase 2: An enigmatic enzyme with diverse functions. Trends Biochem. Sci. **27,** 534, 2002.
8. Melino, G., Candi, E., and Steiner, P.M. Assays for transglutaminases in cell death. Methods Enzymol. **322,** 433, 2000.
9. Volokhina, E.B., Hulshof, R., Haanen, C., and Vermes, I. Tissue transglutaminase mRNA expression in apoptotic cell death. Apoptosis **8,** 679, 2003.
10. Heid, C.A., Stevens, J., Livak, K.J., and Williams, P.M. Real time quantitative PCR. Genome Res. **6,** 986, 1996.
11. Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grinani, F., and Riccardi, C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J. Immunol. Methods **139,** 271, 1991.
12. Engbers-Buijtenhuijs, P., Kamphuis, M., van der Sluijs Veer, G., Haanen, C., Poot, A.A., Feijen, F., and Vermes, I. A novel time-resolved fluorometric assay of anoikis using europium-labelled annexin V in cultured adherent cells. Apoptosis **10,** 429, 2004.
13. Ohtsubo, M., Theodoras, A.M., Schumacher, J., Roberts, J.M., and Pagano, M. Human cyclin E, a nuclear protein essential for the G_1-to-S phase transition. Mol. Cell. Biol. **15,** 2612, 1995.
14. Muller-Tidow, C., Metzger, R., Kugler, K., Diederichs, S., Idos, G., Thomas, M., Dockhorn-Dworniczak, B., Schneider, P.M., Koeffler, H.P., Berdel, W.E., and Serve, H. Cyclin E is the only cyclin-dependent kinase 2-associated cyclin that predicts metastasis and survival in early stage non-small cell lung cancer. Cancer Res. **61,** 647, 2001.
15. Buijtenhuijs, P., Buttafoco, L., Poot, A.A., Daamen, W.F., van Kuppevelt, T.H., Dijkstra, P.J., de Vos, R.A.I., Sterk, L.M.T., Geelkerken, R.H., Feijen, F., and Vermes, I. Tissue engineering of blood vessels: Characterisation of smooth muscle cells for culturing on collagen and elastin based scaffolds. Biotechnol. Appl. Biochem. **39,** 141, 2003.
16. Daamen, W., Veerkamp, J.H., and van Kuppevelt, T.H. Purification of elastin and preparation of matrices for tissue engineering. Ind. Protein **9,** 15, 2001.
17. Heimli, H., Kahler, H., Endresen, M.J., Henriksen, T., and Lyberg, T. A new method for isolation of smooth muscle cells from human umbilical cord arteries. Scand. J. Clin. Lab. Invest. **57,** 21, 1997.
18. Chamley-Campbell, J., Campbell, G.R., and Ross, R. The smooth muscle cell in culture. Physiol. Rev. **59,** 1, 1979.
19. Lefebvre, P., Nusgens, B.V., and Lapiere, C.M. Cultured myofibroblasts display a specific phenotype that differentiates them from fibroblasts and smooth muscle cells. Dermatology **189,** 65, 1994.
20. Salgame, P., Varadhachary, A.S., Primiano, L.L., Fincke, J.E., Muller, S., and Monestier, M. An ELISA for detection of apoptosis. Nucleic Acids Res. **25,** 680, 1997.
21. Hemmilä, I., Dakabu, S., and Mukkala, V.M., Siitari, H., and Lovgren, T. Europium as a label in time-resolved immunofluorometric assays. Anal. Biochem. **137,** 335, 1984.
22. Jones, L.J., Gray, M., Yue, S. T., Haugland, R. P., and Singer, V. L., Sensitive determination of cell number using the CyQuant cell proliferation assay. J. Immunol. Methods **254,** 85, 2001.
23. Olde Damink, L.H.H., Dijkstra, P.J., van Luyn, M.J.A., van Wachem, P.B., Nieuwenhuis, P., and Feijen, J. Purification of elastin and preparation of matrices for tissue engineering. Ind. Protein **9,** 15, 2001.
24. Li, Y., Ma, T., Kniss, D.A., Lasky, L.C., and Yang, S. Effects of filtration seeding on cell density, spatial distribution, and proliferation in nonwoven fibrous matrices. Biotechnol. Prog. **17,** 935, 2001.
25. Smolewski, P., Grabarek, J., Lee, B.W., Johnson, G.L., and Darzynkiewicz, Z. Kinetics of HL-60 cell entry to apoptosis during treatment with TNF-α or camptothecin assayed by the stathmo-apoptosis method. Cytometry **47,** 143, 2002.
26. Pelisek, J., Armeanu, S., and Nikol, S. Quiscence, cell viability, apoptosis and necrosis of smooth muscle cells using different growth inhibitors. Cell Prolif. **34,** 305, 2001.
27. Keyomarsi, K., Tucker, S.L., Buchholz, T.A., Callister, M., Ding, Y., Horogagyi, G.N., Bedrosian, I., Knickerbocker, C., Toyofuku, W., Lowe, M., Herliczek, T.W., and Bacus, S.S. Cyclin E and survival in patients with breast cancer. N. Engl. J. Med. **347,** 1566, 2002.
28. Sutherland, R.L., and Musgrove, E.A. Cyclin E and prognosis in patients with breast cancer. N. Engl. J. Med. **347,** 1546, 2002.
29. Evan, G.I., and Vousden, K.H. Proliferation, cell cycle and apoptosis in cancer. Nature **411,** 342, 2001.
30. Seliktar, D., Nerem, R.M., and Galis, Z.S. Mechanical strain-stimulated remodeling of tissue-engineered blood vessel constructs. Tissue Eng. **9,** 657, 2003.

Address reprint requests to:
I. Vermes, Ph.D.
Department of Clinical Chemistry
Medisch Spectrum Twente Hospital Group
P.O. Box 50,000
7500 KA Enschede, The Netherlands

E-mail: i.vermes@ziekenhuis-mst.nl