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Passage-Dependent Changes in Baboon Endothelial Cells—Relevance to *In Vitro* Aging

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Abstract

In vitro cell culture system is a useful model for aging-related changes in a wide spectrum of biomedical research. In this study, we explored the passage and donor age-dependent changes in baboon macrovascular endothelial cells that are relevant to both *in vitro* cell culture aging models and experiments using cell culture techniques. We collected baboon femoral arterial samples from nine baboons ranging in age from 6 months to 30 years (equivalent to humans approximately 18 months to 90 years of age). We then cultured baboon femoral artery endothelial cells (BFAECs) in standard DMEM medium with 20% fetal calf serum with 1:3 split for subculture. Endothelial functions were documented by morphology, Dil-LDL uptake and expression of eNOS, MCP-1, vWF, VCAM-1, ICAM-1, and E-Selectin with or without cytokine stimulation. Most of the cells became nonmitotic after 30 population doublings, or 10 passages, when they became flattened, enlarged, and senescent. While it took approximately 3 days to reach confluence from three-dilution seeding at early passages (< 6), confluence was not achieved even after 7 days of culture for cells after the 9th or 10th passage. There was a linear decline in eNOS expression with passage. However, this decline was significantly less in endothelial cells from a young baboon (6 months) than those from an old baboon (30 years). While basal expression of adhesion molecules was not changed with passaging, responses to cytokine stimulation appeared to be increased in later passaged cells. Our study has provided evidence for passage-related changes in key endothelial functions. The donor age-related differences in this *in vitro* aging process suggests that *in vitro* endothelial culture can serve as a biomarker for *in vivo* aging. Nonhuman primates can provide a model for investigating such aging-related biological characteristics.

INTRODUCTION

BIOLOGICAL CHANGES during aging and age-associated diseases have increasingly become major health issues in modern society (Hayflick, 2000a, 2000b.) With advancement of medical research, the lifespan of human population has increased steadily. More than 75% of people living in developed countries live more than 75 years. It is anticipated that by 2050, the average life expectancy will reach 82 years (Hayflick, 2000a, 2000b.) However, we still know very little about biological mechanisms that are responsible for age-related changes. On the other hand, enormous progress has been achieved with our understanding of age-associated degenerative diseases including coronary artery disease (CAD), diabetes, hypertension, dementia, osteoporosis, etc. While current medical advancement may be able to slow the progression of many of these diseases, we are still ineffective in slowing or stopping the

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degenerative processes. This is largely due to lack of knowledge about normal biological changes during aging.

One of the major obstacles in aging research is the long study period required to reach an end point in human populations. Some small animal models that have shorter lifespans are useful models, but some fundamental mechanistic differences in key aging process exist between small animals and humans. For example, humans have much longer lifespans, yet, human chromosomes contain only 5–15 kb of the repeat sequence TTAGGG at their ends (telomeres), which dictates the replicative capacity of cells. In contrast, mice have a much shorter lifespan, yet, mice have 20–100 kb of this repeat sequence (Greider, 1996; Morin, 1997; Hemann *et al.*, 2000). However, the mammalian lifespan does seem to be directly associated with replication capability during *in vitro* culture of cells from the species. Human cells can undergo 30–50 population doublings (PDLs) before becoming senescent, whereas mouse cells start proliferative arrest after only 5–10 PDLs in culture. Therefore, an *in vitro* cell culture model rather than an *in vivo* organism model has been used extensively for biomedical and aging research. Although passage-dependent changes in aging research have received close attention, relatively little emphasis has been placed on this important cell behavior in biomedical research that uses cells as hosts or tools. Furthermore, due to the invasiveness of tissue harvesting for cell collection, only limited cell types have been used for investigation of human aging process. It also is difficult to validate *in vitro* cell culture findings in human subjects *in vivo*. In contrast, nonhuman primates, such as baboons, resemble humans anatomically, physiologically, and pathologically (Wang *et al.*, 2004). One of the major advantages is that *in vivo* validation, especially those involving invasive procedures, can be accomplished in well-controlled animal experimental settings. In the current study, we developed an *in vitro* cell culture model in baboons that can be used for follow-up interventional, longitudinal, and mechanistic *in vivo* investigations. We explored the culture passagedependent and donor age-dependent changes in functional endothelial markers in baboons and provided important data for general application of endothelial cells cultured *in vitro* and potentially for an *in vitro* cell aging model.

MATERIALS AND METHODS

Baboon femoral artery endothelial cell culture

Primary baboon femoral artery endothelial cells (BFAECs) used for this study were isolated from nine baboons maintained at the Southwest National Primate Research Center located at the Southwest Foundation for Biomedical Research in San Antonio. Segments of femoral arteries approximately 1–3 cm in length were collected from baboons by sterile methods during necropsy for medical or other research reasons (Table 1). Although some of these baboons were subjected to a high-fat highcholesterol dietary challenge at certain points of their life for approximately 6 months, all baboons were on normal chow diet for at least 6 months prior to necropsy. None of these baboons were purposely exposed to any infectious agents or toxic agents for any research project. Endothelial cells were harvested no longer than 2 h after the vessels were excised. Cell isolation was based on previous reports with modifications (Shi *et al.*, 2004). The arterial sample was placed in a large sterile dish, and the surface was wiped with 2% antibiotic/antimycotic solution (GIBCO-BRL, Grand Island, NY) in PBS. The artery was then gently cannulated at one end with a short blunt needle and flushed with PBS to remove any blood remaining inside the vessel. It was injected with 0.1% collagenase before the other end was cannulated. The closed artery was incubated at 37°C for 15 min for digestion. After completion of digestion, the vessel was massaged gently, flushed with medium, and the released cells were collected by centrifugation and resuspended in medium. The cells were seeded immediately on 1.0% gelatin-coated culture plates. The medium was F-12K supplemented with 20% fetal calf serum (FCS) (GIBCO-BRL), 75 μg/ml EGCS (Sigma, St. Louis, MO), 50 μg/ml heparin, 10 mM HEPES, 2 mM glutamine, and antibiotics. Confluent

cells were passaged by 0.05% trypsin and Versene solution (GIBCO-BRL), and subcultured in a threefold dilution, that is, 1:3 split subculture. The study was approved by Institutional Animal Care and Use Committee (IACUC) of the Southwest Foundation for Biomedical Research.

Cell proliferation measurement

Mitotic index, which is used to monitor the progression of cells through the M phase when chromatin condensation occurs, correlates well with the progression of cells through the M phase and cytokinesis. This method is useful to monitor cell cycle progression (Muehlbauer and Schuler, 2003; Yuan *et al.*, 2003). At the end point of the experiment, endothelial cells cultured in coverslip were washed with cold PBS twice and fixed in 1:3 cold acetic acid:methanol solution. Slides were allowed to air dry for 0.5 to 2 h. Cells were stained with 0.1 to 0.2 mg/liter Giemsa stain for 10 min; the Giemsa stain was gently rinsed from the slides by dipping in PBS solution before the slides were air dried. Slides were observed microscopically for the presence of cells with mitotic changes, which were characterized by condensed nuclear material and a lack of nuclear membrane. The data were presented as a percentage of mitotic cells in the total number of cells observed (N = 1,000).

Morphological examination

Endothelial cells at various stages of passage were monitored daily during culture and phase-contrast pictures were taken during microscopic examination (Leica, Deerfield, IL).

Uptake of Dil-LDL by endothelial cells

Cells were seeded in a Lab-Tek culture chamber to 80–90% confluence. We incubated cells for 4 h in growth medium containing 10 mg/ml fluorescence Dil-labeled LDL (Dil-LDL, Biomedical Technologies, Inc., MA) without supplementing FCS but with 5% calf albumin at 37°C for 4 h. The slides were fixed in 2% formaldehyde solution for 20 min at room temperature before being mounted with SlowFade (Molecular Probes, Eugene, OR) and observed under a fluorescence microscope. Quantification of Dil-LDL up-take was carried out by measuring the fluorescent intensities from three randomly selected visual fields (×200) at the 12, 4, and 8 o'clock axes.

Quantification of cell vWF on endothelial cell surface

Cells at different passages were seeded in 100 μl at a density of 5×10^4/ml in 96-well plates. After reaching 80–90% confluence, the cells were fixed with 2% formalin at room temperature for 20 min and then blocked with 2% BSA in PBS containing 1% H_2O_2 and 0.05% (w/v) Tween-20 at 37°C for 1 h and incubated with antihuman vWF conjugated with HRP (DAKO Cytomation #P0226, Denmark) in a dilution of 1:200. The *o*-phenylenediamine dihydrochloride (OPD, Sigma, Cat# P 9187) was used as the substrate and the absorbance was read at 492 nm after 20 min color development.

eNOS protein quantification by ELISA

After treatment, the cells were collected and lysed in Tris-EDTA buffer with protease inhibitor, and the cell lysates were used for the estimation of eNOS protein levels as described previously. (Wang *et al.*, 2000). Endothelial cells were lysed with lysis buffer, and soluble proteins were extracted by centrifugation. The total soluble protein concentration in the supernatant was determined by the Bradford method (Bio-Rad, Cat# 500-0006). Extracted proteins were diluted to the same concentration for all samples in the ELISA procedures by using an eNOS ELISA kit (R&D systems, Cat. No. DEN00) (Wang *et al.*, 2003). This assay employs the quantitative enzyme immunoassay technique in which a monoclonal antibody specific for human eNOS has been precoated onto a microplate. We have used this method to measure eNOS protein in

baboon arterial wall extracts (Wang *et al.*, 2003). Standards and samples were pipetted into the wells and any eNOS present was bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for human eNOS was added to the wells. Following a wash to remove any unbound antibody–enzyme reagent, a substrate solution was added to the wells and color developed in proportion to the amount of eNOS bound in the initial step. The color development was stopped after 10 min and the intensity of the color was measured. The eNOS concentration of each sample was calculated from a standard curve.

Quantification of cell adhesion molecules on endothelial cell surface

Cells at different passages were seeded in 100 μl at a density of 5×10^4/ml in 96-well plates. After 24 h, the cells were exposed to 10 ng/ml TNF-α, or 10 ng/ml Il-1β or 1 μg/ml LPS (Sigma) for 4 and 24 h. We fixed the cells with 2% formalin at room temperature for 20 min and then blocked with 2% BSA in PBS containing 1% H_2O_2 and 0.05% (w/v) Tween-20 at 37°C for 1 h. The plates were incubated with goat polyclonal antibodies against human E-selectin (R&D Systems, Minneapolis, MN, Cat #BBA19), VCAM-1 (Cat# BBA19) and ICAM-1 (Cat #BBA17) in a dilution of 1:500 at 4°C overnight. After incubation with secondary antibody conjugated with peroxidase (anti-goat IgG Fab_2, Sigma, Cat# A5420) in 1:5,000 at 37°C for 1 h, OPD solution was added. Optical density was read at 492 nm after 20 min color development.

Statistical analysis

Data were expressed as mean ± SEM. A student *t*-test was used to compare the average differences between two groups. ANOVA was applied for comparisons of three groups or more. A two-tailed $P < 0.05$ was regarded as statistically significant. All data reported are representatives of three independent experiments with duplicate sets for each run.

RESULTS

Characteristics of baboon donors

Baboon femoral arterial endothelial cells were collected from baboons necropsied for various reasons. As shown in Table 1, the ages of the animals varied from 6 months to 30 years (approximately equivalent to humans at 18 months to 90 years of age). Three adult female baboons were euthanized for surgical research projects and one for geriatric health reasons. The other five baboons, including two adult females, two young males, and one aged male were euthanized for other health reasons.

Morphological and growth changes of subculturing

While cells from all ages of baboons exhibited the characteristic cobble stone feature of endothelial cells during early passages of culture, during later passages (from ninth passage or after the 27th PDL), cells started to become flattened, enlarged, and less responsive to growth factors (Fig. 1). While it normally took 2–3 days to reach confluence when cells were seeded in a 1:3 split at passages up to 6–7, it often took 5–7 days to reach confluence for cells at the 8–10th passages. For cells older than 10 passages, confluence was never reached within 7 days. In selected typical passages, we found that mitotic indices were not significantly different between the second and fourth passage. However, from the sixth passage, the cultures became less confluent within the same time period, and the cells gradually became flattened and enlarged to a significant degree at the 10th passage (Fig. 1). The one young animal (6 months old) had a more robust growth pattern than four old animals (22 to 33 years old) as indicated by mitotic index values (Table 2) and microscopic observations.

Effect of culture passages on Dil-LDL uptake

Uptake of LDL by endothelial cells is regarded as one of the characteristic features of endothelial cells and is often used to assess the functionality of endothelial cells. In our study, we also evaluated the uptake of Dil-LDL by endothelial cells of selected passages. As shown in Fig. 2, there was no microscopic difference in the capacity of Dil-LDL uptake by endothelial cells of the third (nine PDLs), sixth (18 PDLs), and ninth (27 PDLs) passage. To semiquantitatively analyze the Dil-LDL uptake, we used the ImagePro software to measure the red fluorescent color intensity from three randomly selected visual fields at the 4, 8, and 12 o'clock position of the slide. For example, the intensity of intracellular Dil-LDL in endothelial cells harvested from a 6-month-old baboon decreased approximately 30% (from 496 ± 42 to 342 ± 48 pixels, P = NS). We also noted that endothelial cells from this young baboon appeared to have higher Dil-LDL uptake (496 ± 42) than those of older baboons (e.g., 321 ± 21 for a 30-year-old baboon) at the third passage. The same trend persisted for both the sixth and ninth passages.

Effect of culture passages on vWF expression

We measured vWF expression by anti-vWF antibody conjugated with HRP. No significant loss of vWF expression was observed up to the ninth passage (Fig. 3).

Effects of passage on endothelial cell eNOS production

Endothelial NO production mainly through eNOS enzymatic reaction plays a key role in maintaining functional endothelial integrity. Endothelial dysfunction is often associated with reduced eNOS expression. Therefore, we measured eNOS protein levels from BFAECs cultured to different generations. As shown in Fig. 4, there was almost a linear decline in eNOS protein levels from the 2nd to 10th passage during *in vitro* culture ($P < 0.001$). The eNOS protein levels at the 10th passage (4.6 ± 2.1 pg/μg total protein) were only 13% of the levels at the second to fourth passage (35.7 ± 2.8 pg/μg total protein). Although the consistent decline was uniform for endothelial cells from all ages of baboons, the rate of decline varied according the baboon's age at the time of necropsy. The reduction for a 22-year-old baboon was 93% (from 38.1 pg/μg total proteins at the second to fourth passages to 2.8 pg/μg total protein at the 10th passage), the reduction for endothelial cells collected from 2-year-old baboon was 79% after the same number of PDLs (from 44.3 pg/μg total protein to 9.4 pg/μg total protein). In this sample of only nine baboons, this donor agedependent difference in eNOS expression with culture passage was not statistically significant.

Effect of culture passage on adhesion molecule expression to cytokine stimulation

We measured VCAM-1, ICAM-1, and E-selectin at baseline levels from cells at the third, sixth, and ninth passages. We found little change on the basal level expression in relation to the number of culture passages. In response to stimulation by TNFα and IL-1β, expression of adhesion molecules tended to be stimulated more in cells at the sixth and ninth passages, although the differences did not reach statistical significance (Fig. 5). The change in E-selectin was more prominent at 4 h (Fig. 5A) compared to those at 24 h (Fig. 5B). Response to LPS was not present as we reported previously (Shi *et al.,* 2004). Compared with the endothelial cells collected from a young baboon (19 years old), endothelial cells from a geriatric baboon (30 years old) appeared to have higher increase in CAM expression in response to these cytokine stimulation, although the difference was not statistically significant. For example, at the third to fourth passage, TNFα stimulation increased E-selectin expression by threefold after 4 h exposure in a 30-year-old baboon, but only 2.1 fold in a 19-year-old baboon. The same trends were also seen when cells were stimulated by IL-1β. However, this apparent donor age-related change diminished with the number of culture passages.

DISCUSSION

The results are biologically significant in two aspects. First, the observation of culture passage-dependent reduction in eNOS protein levels is highly relevant to experiments that use cultured endothelial cells for functional studies, because eNOS has a key role in maintaining the normal endothelial function (Cines *et al.,* 1998). A significant reduction in eNOS expression at later passages of the cells would render these endothelial cells less capable in responding to external stimuli. Studies, such as those investigating mechanisms for endothelial apoptosis, would be significantly affected by this passage dependent decline in eNOS. The combination of the donor age dependency and passage-dependent decline in eNOS levels may explain variable outcomes, at times opposite outcomes, for the same treatment when using cultured endothelial cells, or other cells, within or between studies. Hence, valid interpretation of results requires reference to passages and donor ages. Second, our data with baboon endothelial cells have demonstrated the age-dependent changes in several functional features of cultured endothelial cells (Yang *et al.,* 1999; O'Hare *et al.,* 2001), suggesting that baboons may be used for development of *in vitro* cell culture aging model.

In this study, we also found that most baboon endothelial cells may reach senescence during *in vitro* culture after approximately 30 PDLs or 10 passages for adult baboons. This growth capacity is shorter than that of human endothelial cells, which typically reach 40–50 PDLs (or ~15 passages) before cell senescence inhibits confluence (Rubin, 1997; Campisi, 2001). Our findings are consistent with observations of cells obtained from other animals with shorter lifespan than humans (Rubin, 1997; Campisi, 2001; Hornsby, 2003). Baboons have approximately 1/3 of human life expectancy. While the endothelial cells obtained from the 6-month-old and 2-year-old baboons tended to go through more PDLs (~36 PDLs, or 12 passages) before reaching senescence than those of older baboons, the small number of animals in this study makes the interpretation of this observation inconclusive. It is clear, however, that the time required to reach confluence is much prolonged at later culture passages compared to early passages. These findings are consistent with the hypothesis that intrinsic control of cell doubling capacity *in vitro* may reflect intrinsic control of the aging process *in vivo*, and baboon cell culture may be developed to serve as a useful *in vitro* model for aging.

Despite clear deterioration in cell growth capacity and eNOS production, the capacity of Dil-LDL uptake does not appear to be affected by the number of passages. However, endothelial cells of younger baboons tended to have higher uptake of Dil-LDL than those of older baboons and to express fewer adhesion molecules. A study with a larger number of donor animals at different ages will be needed to address this issue statistically.

In addition to vascular aging, aging process in immune system may also play a major role in degenerative vascular diseases, such as inflammatory responses in atherosclerosis. Like all other tissues and organs, information on immune system aging is also limited (Aspinall, 2000). However, nonhuman primates share very similar aging process with humans in relation to telomere shortening in leukocytes (Lee *et al.,* 2002; Baerlocher *et al.,* 2003) cytokine production (Mascarucci *et al.,* 2002), lymphocyte subpopulation differentiation and macrophages (Nam *et al.,* 1998; Lloberas and Celada, 2002; Aspinall, 2003; Damjanovich *et al.,* 2003; Issa, 2003)—all of which appear to decrease with aging although double positive $CD4^+$ and $CD8^+$ T cells appear to increase (Lee *et al.,* 2003). It would be interesting to explore the aging-related changes in immune systems in conjunction with vascular systems with relevance to degenerative vascular diseases. In summary, our study has demonstrated both donor age and *in vitro* cell population doubling dependency in baboon endothelial morphological and functional changes *in vitro*. They highlight that any findings using *in vitro* cell culture, especially endothelial cells, will require reference to the donors' ages and number of passages. Our data also suggest that baboons may serve a good animal model for

in vitro aging studies in which cells can be obtained longitudinally through biopsy and interventional strategies can be applied.

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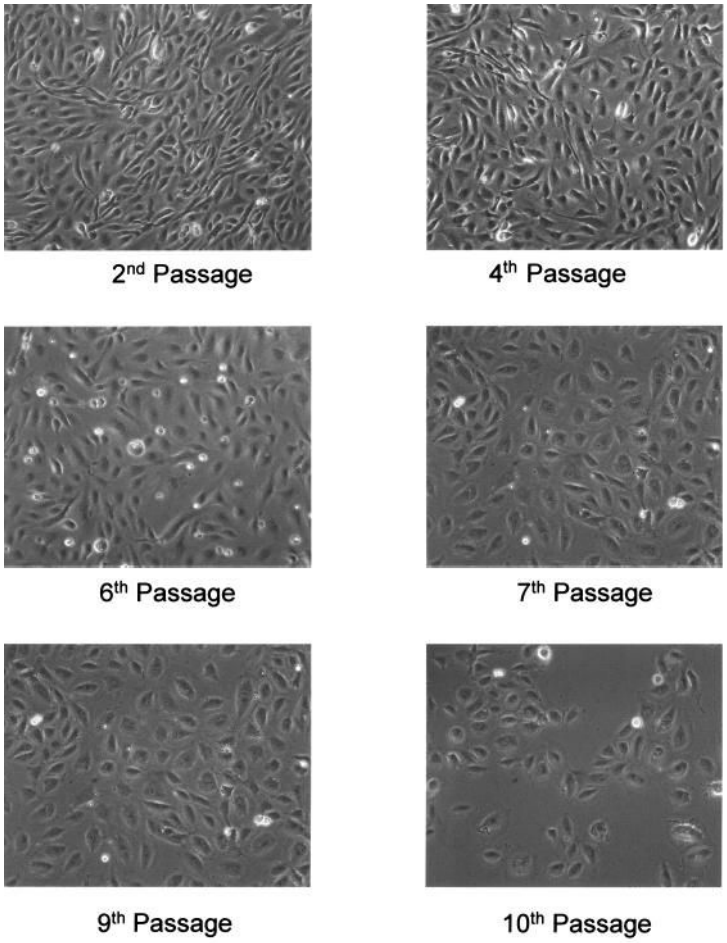


FIG. 1.

Light microscopic observations of baboon femoral arterial endothelial cells (BFAECs, ID 2X0038) cultured to passage numbers 2, 4, 6, 8, 9, and 10, which are equivalent to cell population doublings of 6, 12, 18, 24, 27, and 30. The BFAECs were collected from a 2-year-old baboon (ID# 1 × 16715, Table 1). As shown on the figure, endothelial cells at the 9th or 10th passage had enlarged cytoplasmic volume, and diminished extent of characteristic cobble stone feature.

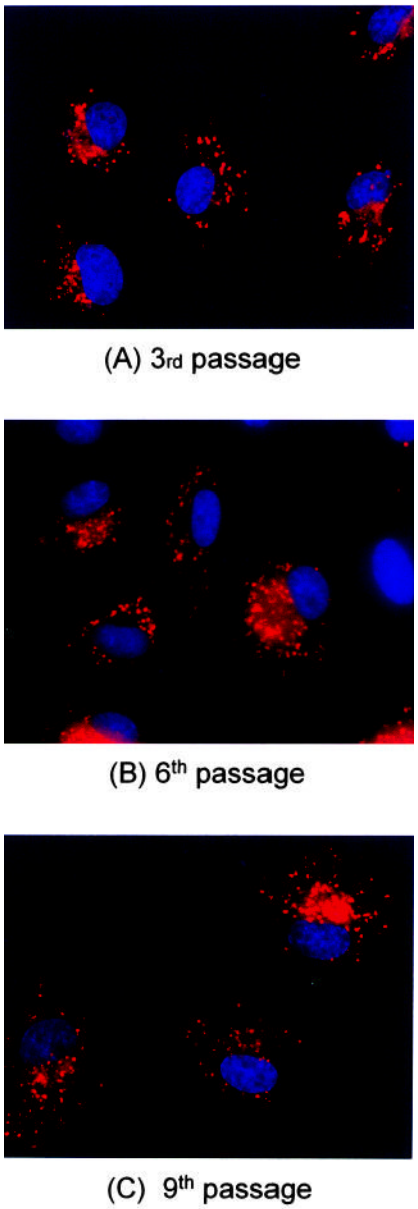


FIG. 2.

Fluorescent microscopic view of BFAECs at the third (**A**), sixth (**B**), and ninth (**C**) passages. The BFAECs were collected from a 6-month-old baboon (ID# 17839, Table 1). The red color was Dil-LDL phagocytosed by endothelial cells and the nuclei were stained blue. The Dil-LDL at a concentration of 10 mg/ml was incubated with endothelial cells for 4 h at 37°C before stained by 1 μg/ml DAPI (blue) and viewed under Nikon upright fluorescence microscoe at magnification of ×400.

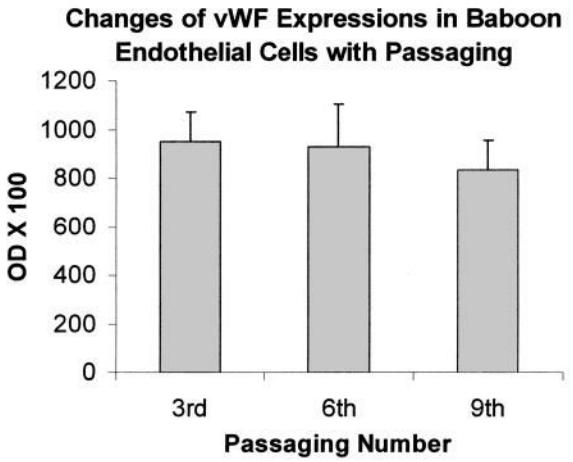


FIG. 3.

Mean ± SEM percent changes in expressions of vWF in baboon endothelial cells at the third, sixth, and ninth passage.

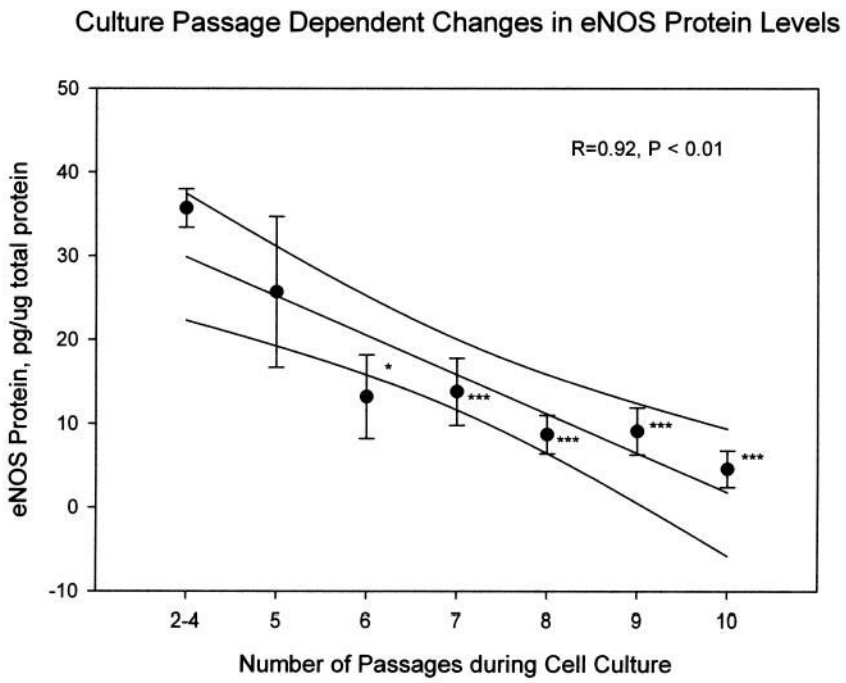
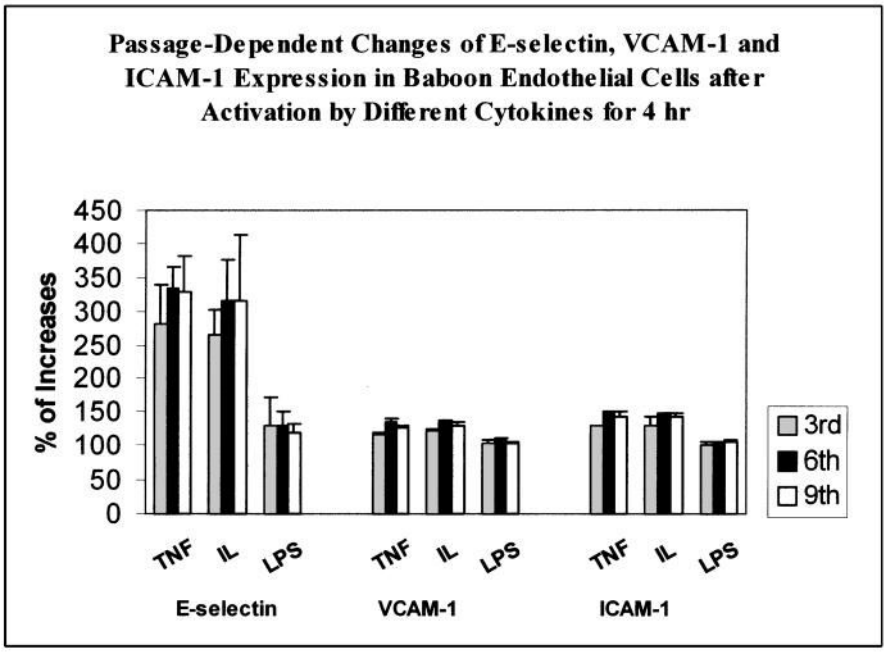


FIG. 4.

Association between the number of culture passages and eNOS protein levels. The data points were the mean ± SEM of endothelial cells from nine baboons. The curved lines above and below the linear fit line mark the 95% confidence interval for the correlation. **P* < 0.05, ****P* < 0.001, by comparison with the eNOS levels at two to four passages.

(A)



(B)

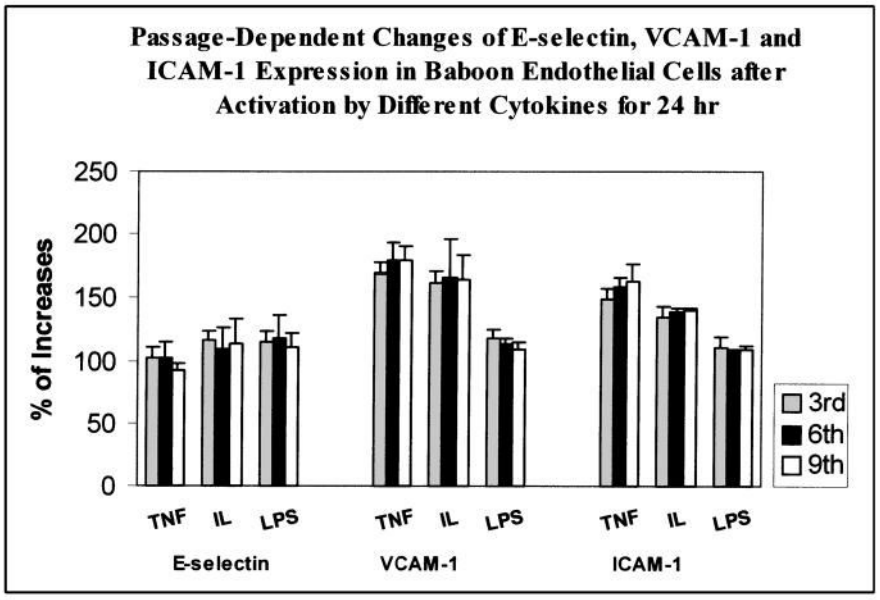


FIG. 5.

Mean ± SEM percent changes in expressions of E-selectin, VCAM-1, and ICAM-1 by baboon endothelial cells under the stimulation of 10 ng/ml TNFα, or 10 ng/mL IL-1β, or 1 μg/ml LPS. BFAECs were cultured up to third, sixth, and ninth passages before they were stimulated by these cytokines for 4 h (**A**) and 24 h (**B**).

Table 1

Animal Information

ID	*Age, years*	*Gender*	*Health status*
1X3707	22.0	F	Healthy
17839	0.5	M	Seizure
1X2713	24	F	Healthy
1X2788	23	F	Healthy
1X0281	19	F	Diabetes
1X1956	33	F	Geriatric
16715	2	M	Pneumonia
2X0038	30	M	Geriatric
1X4756	18	F	Fistula/inflammation

Table 2

Changes in Mitotic Indices of BFAECs During Passaging

ID	*Age, years*	*Third passage*	*Sixth passage*	*Ninth passage*
1X3707	22.0	5.63 ± 0.34	4.93 ± 0.52	1.93 ± 0.14*
1X2788	23	4.87 ± 0.21	5.03 ± 0.19	2.73 ± 0.39*
17893	0.5	7.43 ± 0.48	6.63 ± 0.67	2.13 ± 0.12*
2X0038	30	6.13 ± 0.64	4.23 ± 0.17	0.89 ± 0.44*
1X1956	33	4.01 ± 0.24	3.73 ± 0.25	0.33 ± 0.11*

*$P < 0.05$ compared to third passage. Mitotic index is the number of mitotic cells in 1,000 total cells.