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Effect of serum concentration on adhesion of monocytic THP-1 cells onto cultured EC monolayer and EC-SMC co-culture*

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Abstract: Background: The adhesion of monocytes to the endothelium following accumulation of low-density lipoprotein (LDL) in subendothelial spaces is an important step in the development of intimal hyperplasia in arterially implanted vein grafts and atherosclerosis in both animals and humans. However, it is not well known how serum factors affect the adhesion of monocytes. Methods: We have studied the effect of fetal calf serum (FCS), which we considered a source of LDL, on the adhesion of monocytes to endothelial cells (ECs) by using human monocytic THP-1 cells and both a monolayer of cultured bovine aortic endothelial cells (EC monoculture) and a co-culture with bovine aortic smooth muscle cells (EC-SMC co-culture). Results: It was found that the addition of FCS to the medium greatly affected the adhesion of THP-1 cells, and the higher the concentration of FCS in the medium, the greater the adhesion of THP-1 cells to endothelial cells. Adhesion of THP-1 cells to an EC-SMC co-culture was approximately twofold greater than that to an EC monoculture, and after adhering to endothelial cells, many THP-1 cells trans-migrated into the layer of smooth muscle cells. Conclusion: The results suggest that the elevation of the LDL (cholesterol) level in blood provides a favorable condition for the development of intimal hyperplasia and atherosclerosis by promoting the adhesion of monocytes to the endothelium and their subsequent migration into subendothelial spaces.

Key words: Endothelial cells (ECs), Smooth muscle cells (SMCs), Monocyte, THP-1 cells, Low-density lipoprotein (LDL), Adhesion, Transendothelial migration, Serum concentration

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INTRODUCTION

Adhesion of monocytes to the endothelium and their migration into subendothelial spaces are important steps in the development of intimal hyperplasia in arterially implanted vein grafts and atherosclerosis in both animals and humans (Gerrity, 1981a; 1981b; Zwolak *et al*., 1989; Michison *et al*., 1990; Itoh *et al*., 1994; Stark *et al*., 1997). It occurs preferentially in regions where low-density lipoproteins (LDLs, main carrier of cholesterol in blood) and oxidized low-density lipoproteins (oxLDLs) are accumulated in subendothelial spaces (Schwenke and Carew, 1989; Back *et al*., 1995; Malinauskas *et al*., 1995; Steinberg and Lewis, 1997). Since intimal hyperplasia and atherosclerosis tend to occur under the condition of hypercholesterolemia both in animals and humans, it is considered that the adhesion of monocytes to the endothelium and their subsequent migration into subendothelial spaces are affected by the concentrations of LDL and adhesive proteins in blood. In connection with this, Kinard *et al*.(2001) studied the effects of serum factors on adhesion and transendothelial migration of porcine monocytes and THP-1 cells to a porcine endothelial cell (EC) monolayer and co-culture with smooth muscle cells (SMCs). They showed that the adhesion of THP-1 cells to ECs incubated in serum-containing and serum-free media did not differ significantly. However,

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they did not test the dose effect of the serum. Therefore we have studied the dose effect of fetal calf serum (FCS), which we considered a source of LDL, on the adhesion of monocytes to ECs by using human monocytic THP-1 cells and both a monolayer of cultured bovine aortic endothelial cells (EC monoculture) and a co-culture with smooth muscle cells (EC-SMC co-culture).

MATERIALS AND METHODS

Materials

Bovine aortic ECs and SMCs were purchased from Cell Systems Inc. (Kirkland, WA, USA) and Cell Applications Inc. (San Diego, CA, USA), respectively. Human monocytic leukaemia cell line THP-1 cells were obtained from a repository reference seed stock (depositor: Dr. J. Clarke, AVRI, Pirbright, UK). Calcein-AM used as a fluorescent dye was purchased from Trevigen, Inc. (Gaithersburg, MD, USA). FCS was purchased from Trace Scientific Co., Ltd. (Melbourne, Australia). L-ascorbic acid, fibronectin, penicillin, streptomycin and Iscov's modified Dulbecco's medium (IMDM) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Preparation of EC monocultures and EC-SMC co-cultures

At first, bovine aortic SMCs and ECs were cultivated separately in culture dishes with IMDM containing 10% (v/v) FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% (v/v) CO_2 and 95% (v/v) air humidified atmosphere, and cells at Passages 5~11 were used to prepare EC monocultures and EC-SMC co-cultures.

After obtaining a sufficient number of SMCs, the cells were released from the cell culture dish with a trypsin-EDTA (ethylene diamine tetraacetic acid) solution, centrifuged, and then suspended in IMDM containing 20% (v/v) FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml L-ascorbic acid.

To prepare an EC-SMC co-culture used as a model of an arterial wall, the SMCs were firstly seeded onto 24-well culture plates at 5.0×10^5 cells/cm^2 and cultivated for 3 d in 2 ml of culture medium, and then ECs were seeded at 5.0×10^5 cells/cm^2 directly over the SMCs. Then they were co-cultivated for 3 d in the same culture medium until the ECs became confluent and completely covered the SMCs, as shown in Fig.1 (see Page 627), by the uptake of DiI-labeled acetylated LDL (Ac-LDL), forming an EC-SMC co-culture system. At the same time, monocultures of ECs were also prepared using the same method and cultivated for 3 d until the cells became confluent.

Cultivation of THP-1 cells

Human monocytic leukaemia cell line THP-1 cells were cultivated in suspension in IMDM containing 10% FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin at 37 °C in 5% CO_2 and 95% air humidified atmosphere.

Preparation of fluorescent THP-1 cells

To visualize the THP-1 cells adherent to the EC monoculture and the EC-SMC co-culture, the THP-1 cells were labeled with a fluorescent dye, calcein-AM, by incubating 2.0×10^6 cells in 4 ml of IMDM containing 10% FCS and 40 μl of calcein-AM to make a final calcein-AM concentration of 10 μmol/L at 37 °C for 1 h. Dye loading was stopped by adding 4 ml of cold IMDM containing 10% FCS to the suspension and then centrifuging. Fluorescence-labeled cells were resuspended in 4 ml of IMDM containing 10% FCS and prepared for use in adhesion experiments.

Procedures for adhesion experiments of THP-1 cells

To investigate the effect of the concentration of serum added to a cell culture medium as a source of LDLs on the adhesion of THP-1 cells to the endothelium, both EC monocultures and EC-SMC co-cultures were divided into 3 groups and the media were discarded. The cells in the EC monoculture and EC-SMC co-culture were rinsed 3 times with 1 ml of phosphate-buffered saline (PBS, pH 7.3) to wash out the FCS. Then 4 ml of new culture medium containing antibiotics and FCS either at a concentration (v/v) of 20%, 1% or 0.1% was put in each dish and they were incubated overnight at 37 °C in 5% CO_2 and 95% air humidified atmosphere to get ready for use in adhesion experiments of THP-1 cells.

The EC monocultures and EC-SMC co-cultures were taken out of the incubator. Then 20~30 μl of suspension of calcein-AM-labeled THP-1 cells con-

taining 1.0×10^4 cells was added to each culture dish and they were incubated for a period of either 30 min, 1 h, 2 h or 4 h at 37 °C in 5% CO_2 and 95% air humidified atmosphere. After that, the suspension of THP-1 cells in each dish was discarded, and the monoculture and co-culture were gently washed three times with 1 ml of PBS at pH 7.3 in order to remove non-adherent THP-1 cells. The number of adherent THP-1 cells was determined by measuring the fluorescence intensity of the adherent THP-1 cells using a Fluoroskan Asent® FL (Thermo Labsystem, Helsinki, Finland) and also by counting the number of adherent fluorescence-labeled THP-1 cells that were seen under a fluorescence microscope (Nikon, Japan) which encompassed a surface area of 0.314 mm^2.

Measurements were carried out at 6 different areas and the results were expressed as the number of THP-1 cells per mm^2.

Observation of THP-1 cells transendothelially migrated into the SMC layer

To visualize the THP-1 cells transendothelially migrated into the SMC layer, a special EC-SMC co-culture was prepared by seeding SMCs on the inner surface of an expanded PTFE (polytetrafluoroethylene) graft at 2.0×10^6 cells/cm^2 by means of pressure infusion of a suspension of SMCs using a syringe. The PTFE graft, 3.0 mm i.d. (inner diameter) and 1.0 cm long, was treated with 70% (v/v) ethanol and coated with 0.01% (w/v) fibronectin, an adhesive molecule. The cells were cultivated for 7 d in 40 ml of IMDM containing 20% FCS, 100 IU/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml L-ascorbic acid at 37 °C in 95% air and 5% CO_2 humidified atmosphere. Then, ECs were seeded directly over the SMCs in the same manner as that of SMCs, and they were co-cultivated for 7 d in 40 ml of the same culture medium until the ECs became confluent and completely covered the SMCs, forming an EC-SMC co-cultivated hybrid vascular graft. Then 20~30 μl of a suspension of THP-1 cells containing 1.0×10^4 cells was added to the culture dish containing the hybrid graft and incubated for 4 h at 37 °C in 5% CO_2 and 95% air humidified atmosphere. After that, the suspension of THP-1 cells in the dish was discarded, and the hybrid graft was gently washed three times with PBS at pH 7.3 in order to remove non-adherent THP-1 cells. The cells of the hybrid graft were fixed with a 4% (w/v) formaldehyde solution and embedded in paraffin. Then the graft inbedded in paraffin was sliced into 5-μm thick sections, stained with a hematoxylin-eosin (HE) stain, and observed under a light microscope.

Statistical analysis

The result was expressed as a mean±*SD*. The significance in the difference between the means of any two groups was assessed by Student's *t*-test. In experiments involving multiple groups, *P*-values between the means of any two groups were calculated by the analysis of variance (ANOVA) using Scheffe's test. Differences were considered statistically significant if $P<0.05$.

RESULTS

Effect of serum concentration on adhesion of THP-1 cells

Fig.2 (see Page 627) shows the photographs of calcein-AM labeled THP-1 cells adhered to ECs cultivated in media containing serum at 3 different volume concentrations (0.1%, 1% and 20%) after incubation for 1 h. As is evident from Fig.2, the adhesion of THP-1 cells was affected by the concentration of serum in the medium, and it was the greatest with the cells incubated with a medium containing serum at 20%, among the three concentrations tested. It was also found that the adhesion of THP-1 cells was affected by the duration of incubation as shown in Figs.3a and 3b respectively, in terms of fluorescence intensity and the number of THP-1 cells. As shown in Figs.2 and 3, both fluorescence intensity and the number of THP-1 cells that appeared to be adherent to the surface of ECs in the monoculture were the highest at a serum concentration of 20% at any time, and they appeared to decrease over time. Fig.4 shows the results obtained with EC-SMC co-cultures. The tendency of THP-1 cell adhesion in EC-SMC co-culture was almost the same as that in the case of EC monocultures, the highest appearing at a serum concentration of 20% at any time. However, the number of THP-1 cells actually adherent to the surface of endothelial cells was approximately two times higher than that found in the EC monocultures.

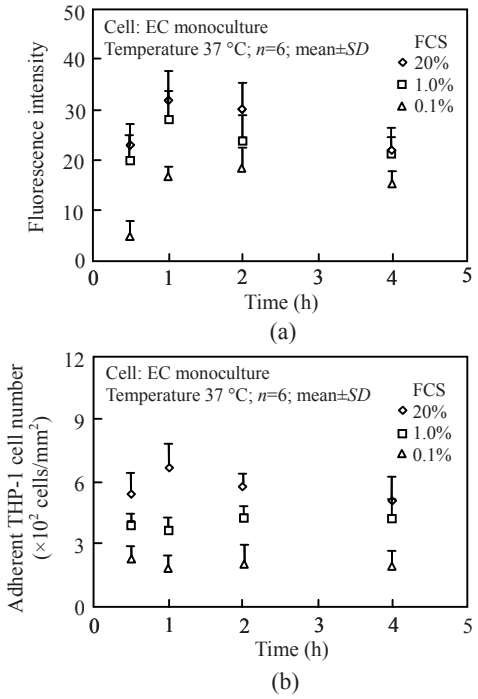

Fig.3 Measured fluorescence intensity (a) and the number (b) of calcein-AM-labeled THP-1 cells adherent to an EC monoculture after incubation of the cells for 1~4 h in media containing FCS at three different volume concentrations of 0.1%, 1% and 20%, showing the effect of FCS concentration on the adhesion of THP-1 cells and time course of the adhesion

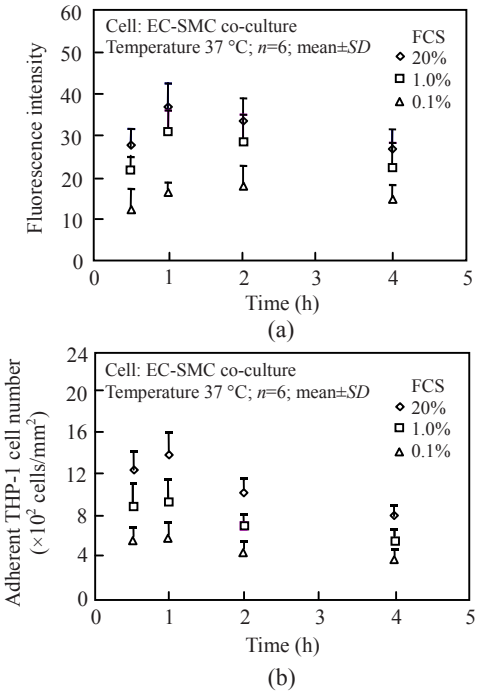

Fig.4 Measured fluorescence intensity (a) and the number (b) of calcein-AM-labeled THP-1 cells adherent to an EC-SMC co-culture after incubation of the cells for 1~4 h in media containing FCS at three different volume concentrations of 0.1%, 1% and 20%, showing the effect of FCS concentration on the adhesion of THP-1 cells and time course of the adhesion

Effect of the presence of smooth muscle cells on adhesion of THP-1 cells

To investigate the effect of the presence of SMCs under the ECs in EC-SMC co-cultures on the adhesion of THP-1 cells to the surface of ECs, we counted the number of adherent THP-1 cells in both the EC monoculture and EC-SMC co-culture after 1-hour incubation with THP-1 cells in a culture medium containing serum at three different concentrations. Fig.5 summarizes the results. As evident from the figure, the adhesion of THP-1 cells was greatly enhanced by the presence of SMCs under a monolayer of ECs. At any serum concentration, the number of adherent cells in the EC-SMC co-culture was approximately twofold of that in the EC monoculture and the difference was statistically significant.

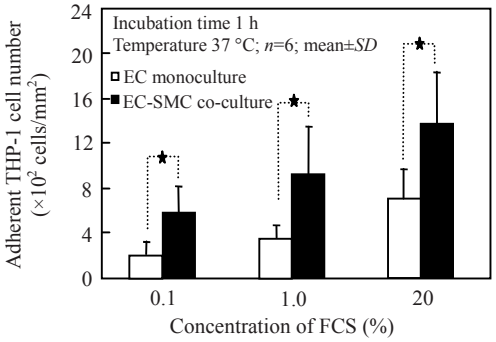

Fig.5 Comparison of the number of THP-1 cells adherent to an EC monoculture and EC-SMC co-culture after incubation of the cells with it for 1 h in media containing FCS at three different volume concentrations of 0.1%, 1% and 20%, showing the effect of FCS concentration. ⋆Significantly different (P<0.01)

Transendothelial migration of THP-1 cells

To find out whether the migration of THP-1 cells into subendothelial spaces occurs upon adhesion to ECs, we prepared an EC-SMC co-culture on an expanded PTFE artificial graft, and after finishing adhesion experiments, we made histological specimens of the hybrid graft and observed them under the microscope. Fig.6 shows the crosssection of the hybrid graft obtained after incubation with THP-1 cells for 1 h. It was found that after adhering to ECs, many THP-1 cells migrated across the monolayer of ECs and accumulated in subendothelial spaces.

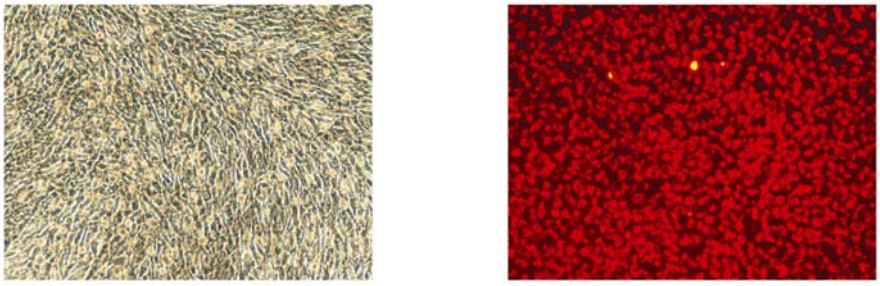

Fig.1 En face microscopic photographs of EC-SMC co-cultures prepared by directly seeding bovine aortic endothelial cells on layers of smooth muscle cells and co-cultivating them. (a) EC-SMC co-culture observed under a phase contrast microscope; (b) EC-SMC co-culture observed under a fluorescent microscope, showing a monolayer of ECs that took up fluorescent dye-labeled acetylated LDL (DiI-labeled Ac-LDL; 5 μg/ml), which is known to be taken up specifically by ECs

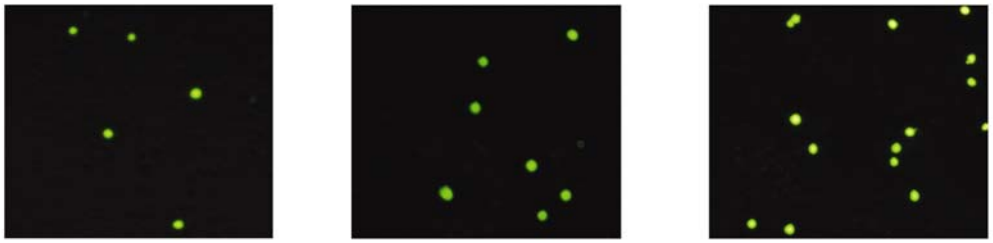

Fig.2 En face fluorescence microscopic photographs of calcein-AM-labeled THP-1 cells adherent to an EC monoculture after incubation of the cells for 1 h in media containing FCS at three different volume concentrations of 0.1% (a), 1% (b) and 20% (c)

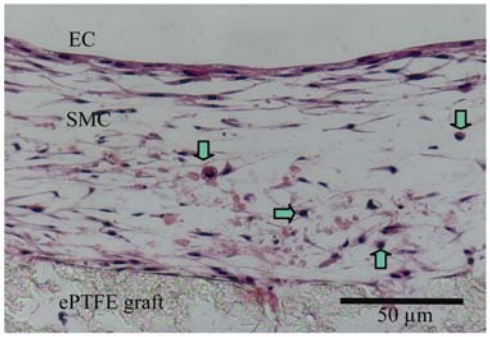

Fig.6 A light-microscopic photograph of the crosssection of an EC-SMC co-culture prepared on an expanded PTFE (ePTFE) graft that underwent 1-h incubation with THP-1 cells in a medium containing FCS at a volume concentration of 20% and was stained with hematoxylin-eosin (HE). Note that after adhering to endothelial cells (ECs), many THP-1 cells migrated across the monolayer of ECs into the smooth muscle cell (SMC) layer as shown by the arrows

DISCUSSION

It is known that under normal physiologic conditions, monocytes do not easily adhere to the endothelium. Adhesion of monocytes occurs following accumulation of LDLs in subendothelial spaces. Since LDLs are present in serum, we considered that the adhesion of THP-1 cells is affected by the concentration of serum in a medium. Therefore in our present study, we tested three different FCS concentrations and obtained a result very different from that of Kinard *et al*.(2001). Adhesion of THP-1 cells to both the EC monoculture and EC-SMC co-culture increased with an increase in the concentration of serum in the medium. A difference was also seen in the time course of adhesion. Kinard *et al*.(2001) showed that the adhesion of THP-1 cells increased over time and reached a constant value after 6 h, whereas our results showed a maximum after 2 h and then a gradual decrease over time. Kinard *et al*.(2001) also tested the adhesion of THP-1 cells to ECs in monoculture or in co-culture with SMCs preincubated in the presence or absence of native LDL, oxLDL or LPC (lysophosphatidylcholine, a component of oxLDL). They found that LPC in a monoculture and LPC and oxLDL in a co-culture enhanced the adhe-

sion of THP-1 cells. Berliner *et al*.(1990) also showed that oxLDL enhanced the adhesion of monocytes to cultured rabbit aortic ECs by the action of LCP. In their additional experiments, Kinard *et al*.(2001) found that monocyte chemoattractant protein-1 (MCP-1) produced by SMCs is implicated in both enhanced adhesion and transendothelial migration of THP-1 cells in the same manner as LPC. Cushing *et al*.(1990) demonstrated that minimally oxidized LDLs, but not native LDLs, induced the production of MCP-1 in vascular wall cells such as ECs and SMCs.

In our present study, the number of THP-1 cells adherent to ECs increased with an increase in the concentration of FCS in the medium in both monoculture and co-culture with SMCs. If it is true that native LDLs do not affect the adhesion of THP-1 cells as shown by Kinard *et al*.(2001), it is likely that oxLDLs modified from native LDLs after being taken up by the ECs during preincubation are responsible for this phenomenon. The decrease in the number of the adherent THP-1 cells that occurred beyond 2-h incubation might be caused either by the decrease in the amount of LDLs in the medium due to the uptake of LDLs and oxLDLs by non-adherent THP-cells or by the disappearance of adhesive molecules. In our present study, we did not try experiments with a serum-free medium since Cai *et al*.(2004) showed that an insufficient supply of serum causes apoptosis of THP-1 cells.

We also found that the adhesion of THP-1 cells was greatly enhanced by the presence of SMCs. The number of THP-1 cells adherent to the ECs of an EC-SMC co-culture was approximately twofold of that of the EC monoculture. Our results are quite the same as those obtained by Kinard *et al*.(2001) in the presence of oxLDL.

In our experiments, it was observed that the number of THP-1 cells adherent to the ECs of the EC-SMC co-culture decreased greatly over time, although the intensity of fluorescence emitted by calcein-AM-labeled THP-1 cells adhering to and presumably taken up in part by the EC-SMC co-culture did not decrease so much. Therefore we prepared histological specimens of the co-culture and examined them under a light microscope. It was found that there were many THP-1 cells in the subendothelial cell layer composed of SMCs, suggesting that most of the THP-1 cells migrated into the subendothelial SMC layer upon their adhesion to ECs. It is likely that LDLs in the serum changed to oxLDL after being taken up by the ECs. Then they were transported to the SMC layer underlying the ECs, and induced the migration of THP-1 cells.

In summary, we studied the effect of the concentration of FCS added to the culture medium on adhesion of human monocytic THP-1 cells to an EC monoculture and an EC-SMC co-culture and found that the higher the concentration of serum in the medium, the greater the adhesion of THP-1 cells to ECs, suggesting that elevation of the LDL (cholesterol) level in blood provides a favorable condition for the development of intimal hyperplasia and atherosclerosis.

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