# Human Arterial Smooth Muscle Cell Proliferation in Diabetes

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**In the present study, we focus on the proliferation of human arterial smooth muscle cells (SMCs) from NIDDM patients (DM-SMCs) to clarify the reactivity to the growth factor(s) in fetal calf serum (FCS) and the factor(s) secreted by T-cells. The proliferation of DM-SMCs was significantly greater than SMCs from nondiabetic patients (nonDM-SMC). DM-SMC conditioned medium (DM-condMed) increased the growth of nonDM-SMCs. These results suggest that the growth factor is secreted from DM-SMCs as an autocrine system, which increases the proliferation of nonDM-SMCs. T-cells increased DNA synthesis of SMCs, and DM-SMCs strikingly reacted to T-cells. The present results support a function of T-cells in stimulating SMC growth. In conclusion, human arterial SMC proliferation is increased in diabetes in the same fashion as in experimentally induced diabetes in animals through responses to growth factors and an increased autocrine system. These results provide a mechanism for the increase in atherosclerotic disease in diabetes. *Diabetes* 45 (Suppl. 3):S114–S116, 1996**

The proliferation of smooth muscle cells (SMCs) has been observed in the arterial intima of atherosclerotic lesions (1,2). This is the important event of atherosclerosis formation. It has been suggested in diabetic animals that proliferation of SMCs is increased (3–6). The presence of T-cells in atherosclerotic lesions has also been shown (7). It is reported that T-cells secrete growth factors and increase the proliferation of arterial SMCs in vitro (8). In the present study, we focus on the proliferation of human arterial SMCs to clarify the relationship between growth factor(s) in fetal calf serum (FCS) and the factor(s) secreted by T-cells.

## RESEARCH DESIGN AND METHODS

**Materials and reagents.** FCS, minimum essential medium (MEM), and RPMI-1640 culture media were purchased from Gibco/BRL (Grand Island, NY).

A DNA assay kit (Cell Proliferation Assay) using 5-bromo-2′-deoxyuridine (BrdU) was purchased from Amersham (Arlington Heights, IL). The 12- and 24-well multiwell culture dishes and petri culture dishes (60 mm in diameter) were purchased from Nunc (Roskilde, Denmark). The human T-cell recovery column was obtained from Biotex (Atlanta, GA). Transwell culture dishes (6.5 mm in diameter and 0.4 μm in pore size) were purchased from Costar (Cambridge, MA).

**Subjects.** Four NIDDM patients were studied and four nondiabetic patients were used as control subjects. The control subjects were not obese and had no hyperlipidemia or other metabolic disorders, except for hypertension in nondiabetic patient 1 (Table 1). All had early gastric cancer and had undergone gastrectomy. The lesion was limited to a small area, and no invasion into surrounding areas was observed.

**Culture of human arterial SMCs.** The right or left gastric artery was obtained after the gastrectomy. Explants were aseptically prepared and attached to the petri culture dishes by the methods of Ross (9). MEM-FCS (10%; 3 ml) was added to each dish, and the dish was incubated in a humidified $CO_2$ incubator (5% $CO_2$ and 95% air) at 37°C. The culture medium was replaced twice per week. SMCs from NIDDM patients (DM-SMCs) and from nondiabetic patients (nonDM-SMCs) were used in the experiments passages 2 through 9.

**Effect of diabetes on SMC proliferation.** An experiment was designed to check the response of SMC to FCS. The number of DM-SMCs and nonDM-SMCs at the same passages was adjusted to $0.5 \times 10^4$/well in 12-well multiwell dishes in MEM-FCS (0.5%). Two kinds of FCS concentrations (5 and 10%, v/v) were prepared, and 2 ml of each medium was added into the wells. The number of SMCs was measured on the 1st, 3rd, 6th, 9th, and 12th days.

Another experiment studied the effect of conditioned medium of cultured DM-SMCs on the growth of nonDM-SMCs. The number of DM-SMCs at the passages 6 through 9 was adjusted to $3 \times 10^5$/dish (60 mm in diameter), and the cells were cultured with MEM-FCS (10%). After 3 days of culture, the medium was designated as the conditioned medium of DM-SMC (DM-condMed). The experimental medium was prepared by the addition of DM-condMed into MEM-FCS (10%), being 20% (v/v). NonDM-SMCs ($0.5 \times 10^4$/well) were cultured with 2 ml experimental medium containing DM-condMed. The number of cells was measured on the 1st, 3rd, 6th, 9th, and 12th days.

**Effect of T-cells on SMC proliferation**

***Preparation of T-cells.*** Mixed mononuclear cells were prepared from venous blood of normal volunteers by the methods of Boyum using Ficoll-paque (10). These cells were incubated with RPMI-1640 in petri culture dishes for 2 h. Nonadherent cells were rinsed and resuspended in phosphate-buffered solution (PBS). This cell suspension was applied on a T-cell recovery column. The recovered T-cells were suspended in MEM-FCS (10%).

**Effect of T-cells on the SMC growth.** SMCs ($1 \times 10^4$/well) were seeded into 24-well multiwell dishes and incubated with MEM-containing 0.5% FCS to fix the cells at $G_0$ in the cell cycle. The medium was removed 24 h later. SMCs in the multiwell dish was cocultured with T-cells ($4 \times 10^5$/well) in 1 ml MEM-containing 10% FCS in the upper chamber. This coculture of SMC with T-cells was performed transmembrane to avoid direct contact for 24 h. The transmembrane and T-cells in the upper chamber were removed, and the culture medium was replaced with fresh MEM-FCS (10%). NonDM-SMCs and DM-SMCs were incubated for 24 h with BrdU. BrdU incorporated into DNA was measured in SMCs by enzyme-linked immunosorbent assay.

## RESULTS AND DISCUSSION

The increase in the number of SMCs during the 12 days of culture is shown in Fig. 1. The growth of both nonDM-SMCs

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Accepted for publication 26 September 1995.

BrdU, 5-bromo-2′-deoxyuridine; DM-condMed, conditioned medium of smooth muscle cells from diabetic patients; FCS, fetal calf serum; FGF, fibroblast growth factor; MEM, minimal essential medium; PBS, phosphate-buffered solution; PDGF, platelet-derived growth factor; SMC, smooth muscle cell.



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TABLE 1
Subjects

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Age (years) | 52 | 47 | 67 | 59 | 74 | 61 | 50 | 45 |
| Sex | M | M | M | M | M | M | M | M |
| Fasting blood glucose (mg/dl) | 107 | 128 | 120 | 116 | 90 | 99 | 92 | 100 |
| $HbA_{1c}$ (%) | 8.3 | NE | 8.1 | NE | | | | |
| Duration of diabetes | 8 years | 0.5 months | 13 years | 10 years | | | | |
| Therapy | OHA | — | OHA | OHA | | | | |
| Complication | SDR | — | — | — | | | | |

NE, not examined; OHA, oral hypoglycemic agent; SDR, simple diabetic retinopathy.

and DM-SMCs was significantly greater in the culture with 10% FCS than with 5% FCS. Both cell types responded to growth factors in FCS. The proliferation of DM-SMCs was significantly greater than that of nonDM-SMCs, as previously reported (1–4). Kawano et al. (11) reported that DM-SMCs expressed more receptors for platelet-derived growth factor (PDGF) B-chain than did the nonDM-SMCs. It is suggested here that DM-SMCs reacted to the growth factor(s) of FCS more strikingly than the nonDM-SMCs through a similar mechanism of increased growth factor receptors.

The effect of DM-condMed on the growth of nonDM-SMCa is shown in Figs. 2 and 3. The growth of DM-SMCs was greater than nonDM-SMCs, as shown in Fig. 1. The proliferation of nonDM-SMCs was a greater increase with the addition of DM-condMed during 6 days of culture compared with nonDM-SMCs cultured without DM-condMed. The number of cells after 12 days of culture, including data from Fig. 2, is shown in Fig. 3. The number of nonDM-SMCs was significantly increased ($P < 0.03$) by the addition of DM-condMed compared with nonDM-SMC without DM-condMed. These results suggest that some factor was secreted by DM-SMCs to a greater extent than by nonDM-SMCs. Such factors might include the SMC-derived growth factor (12) or fibroblast growth factor (FGF) (13,14). These results suggest that the growth factor is secreted from DM-SMCs as an autocrine system, which increases the proliferation of nonDM-SMCs. There are two possibilities to explain the increased proliferation of diabetic SMCs; one is the increased reactivity to growth factor(s), and the other is the increased function of an autocrine system.

The effect of T-cells on DNA synthesis of nonDM-SMCs is shown in Fig. 4. DNA synthesis of nonDM-SMCs was signif-

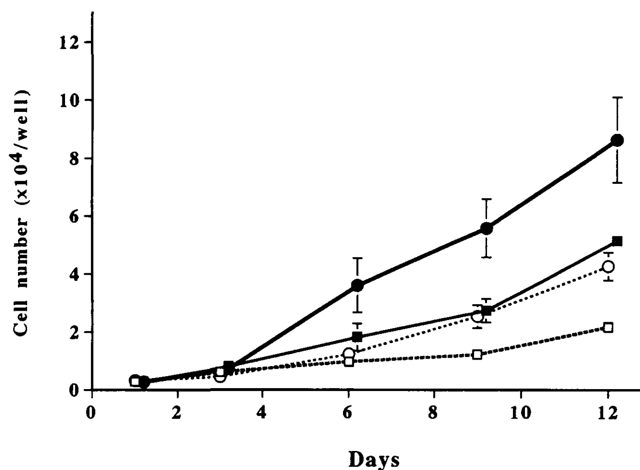

**FIG. 1. The increase of human arterial SMCs during 12-day culture with 5 and 10% FCS. DM-SMCs and nonDM-SMCs were used in the experiments passages 2 through 9. At the start of the experiment (day 0), the cell number was adjusted to 0.5 × $10^4$/well in 12-well multiwell dishes. Two different experimental media were prepared from MEM containing 5 and 10% FCS. The media were exchanged every 3 days. The number of SMCs was calculated on the 1st, 3rd, 6th, 9th, and 12th days. The data are shown as means ± SE calculated from five different experiments. Statistical significance was determined using an unpaired *t* test. DM-SMCs cultured with 10% FCS (●) proliferated significantly more than DM-SMCs cultured with 5% FCS (○) on the 6th day ($P < 0.05$), 9th day ($P < 0.03$), and 12th day ($P < 0.03$), and more than nonDM-SMCs cultured with 10% FCS (■) on the 9th day ($P < 0.04$) and 12th day ($P < 0.05$). DM-SMCs cultured with 5% of FCS (○) also increased significantly more than nonDM-SMCs cultured with 5% (□) FCS on the 9th day ($P < 0.02$) and 12th day ($P < 0.01$). These results indicate that DM-SMCs proliferated much more than nonDM-SMCs.**

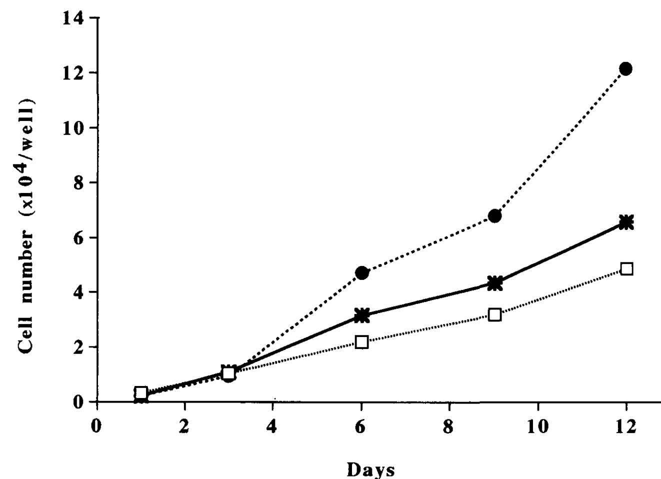

**FIG. 2. The sequential changes in the number of SMCs in culture with DM-condMed. DM-SMCs (3 × $10^5$/dish, ●) at passages 6 through 9 were cultured with 10% FCS containing MEM. The media for 3-day culture were prepared as DM-condMed. The DM-condMed was added into the culture media, and the concentration was adjusted to 20% (v/v). This conditioned medium was used in the nonDM-SMC culture (□). The cells were prepared in the 12-well multiwell dishes (0.5 × $10^4$/well) and added with 1 ml of MEM-FCS (10%) supplemented with DM-condMed (*). The number of cells was calculated on the 1st, 3rd, 6th, 9th, and 12th days of the culture. The data are shown as mean values calculated from two different experiments.**

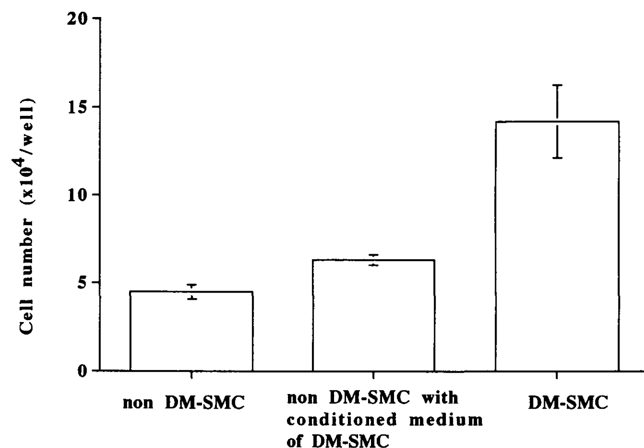

**FIG. 3. The effect of DM-condMed on the proliferation of nonDM-SMCs. The arrangement of SMCs was the same as in the experiment shown in Fig. 2. The number of SMCs was calculated on the 12th day of the experiment, after the addition of DM-condMed. The results are shown as means ± SE calculated from three different experiments including a part of the data in Fig. 2. There is a significant difference ($P < 0.03$) between the cell number of nonDM-SMCs cultured with and without DM-condMed.**

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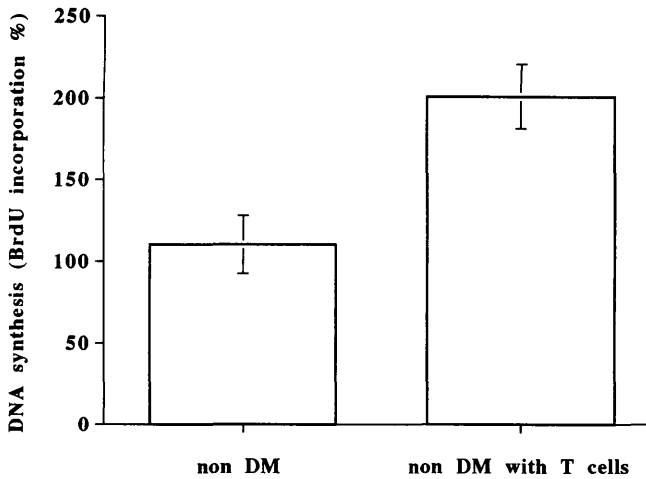


FIG. 4. The effect of T-cells on DNA synthesis of nonDM-SMCs. Methods are described in the text. Values for BrdU incorporation are shown as means ± SE calculated from six different experiments. There is a significant difference ($P < 0.01$) between DNA synthesis of nonDM-SMCs incubated with and without T-cells.

icantly increased ($P < 0.01$) by coculture with T-cells. DNA synthesis of nonDM-SMCs and DM-SMCs in the coculture with T-cells was concomitantly studied as shown in Fig. 5. The presence of T-cells increased DNA synthesis of SMCs,

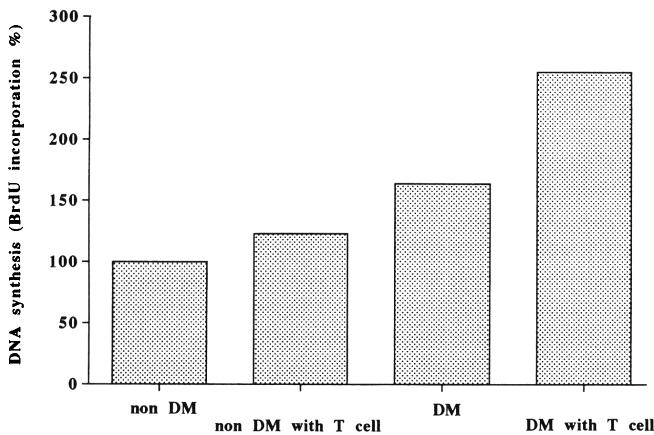


FIG. 5. The effect of T-cells on DNA synthesis of DM-SMCs. The preparation of T-cells was the same as the experiment shown in Fig. 4. The results are shown as mean values of two different experiments, including the results of Fig. 4. DM-SMCs reacted strikingly to T-cells.

and DM-SMCs reacted strikingly to T-cells. It has been reported that T-cells secrete basic FGF and heparin-binding epidermal growth factor–like growth factor (8). The present results support a function of T-cells in stimulating SMC growth. DM-SMC proliferation would be increased.

In conclusion, human arterial SMC proliferation is increased in diabetes in the same fashion as in experimentally induced diabetes in animals through responses to growth factors and an increased autocrine system. These results provide a mechanism for the increase in atherosclerotic diseases in diabetes.

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