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Inhibitors of Phospholipase Promote Apoptosis of Human Endothelial Cells

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In order to understand the signal transduction system that regulates apoptosis of human umbilical vein endothelial cells (HUVEC), we investigated the effects of inhibitors of the activity of phospholipases. All three tested inhibitors of phospholipase A_2 (PLA_2), namely, manoalide, 3-(4-octadecyl)benzoylacrylic acid (OBAA), and oleyoxyethylphosphorylcholine (OOPC), induced apoptotic cell death of HUVEC. After 16 h of treatment, almost all of the cells had disintegrated into apoptotic bodies, and DNA ladders characteristic of apoptotic cell death were clearly observed upon analysis of DNA on agarose gels. The release of arachidonic acid from the cells that had been treated with manoalide, OBAA or OOPC (at the same concentrations as those at which these compounds induced apoptosis) was inhibited. We also studied the effects of two inhibitors of phosphatidylinositol-specific phospholipase C (PLC), U73122, and compound 48/80. Both compounds promoted the apoptosis of HUVEC. After 16 h of treatment, few cells remained intact, and DNA fragmentation was clearly detectable after only 12 h. Quantitation of inositol released from cells treated with U73122 and compound 48/80 showed that the release of inositol was blocked. By contrast, U73343, a similar aminosteroid that does not inactivate PLC, had no such effects. Our results suggest that PLA_2 and phosphatidylinositol-specific PLC might be involved in the signaling pathway of apoptosis in HUVEC, and that the metabolism of arachidonic acid and of inositol might play important roles in the present apoptotic signal-transduction system.

Key words: apoptosis, endothelial cell, phospholipase, signal transduction.

Apoptosis, namely, programmed cell death, is an endogenous mechanism for cell suicide that can be selectively induced by various stimuli, which tend to be specific for specific types of cell. When this mechanism is impaired, significant diseases such as arteriosclerosis and hemangioma are likely to occur (*1*). To understand apoptosis we must characterize its molecular mechanism. Distinct apoptotic signaling pathways (*2*) seem to be involved in different types of cell. Furthermore, even in a given type of cell, there are several pathways for transduction of the apoptotic signal depending on the stimulus (*3, 4*).

Endothelial cells play important roles in the formation of blood vessels and their degeneration (*5*). Therefore, apoptosis of endothelial cells may have a positive role in the control of vascular degeneration. In our previous papers we reported the induction of apoptosis in HUVEC by deprivation of fibroblast growth factor (FGF) and serum, as well as the involvement of protein kinase C in the signal transduction of this apoptotic process (*6, 7*). Many experimental results indicate that the activation of protein kinase C is intimately related to the activity of phospholipase A_2 (PLA_2) (*8-10*), and PLA_2 is thought to play a key role in cell signaling *via* the release of arachidonic acid from glycerophospholipids (*11-13*). Phospholipase C (PLC) is another important enzyme in intracellular signal transduction (*14-16*); it plays a critical role in the regulation of cell proliferation (*17-20*). However, we do not know anything about the roles of PLA_2 and PLC in apoptosis of HUVEC. In this study, we investigated the effects of PLA_2 and PLC on apoptosis of HUVEC using specific inhibitors of PLA_2 and PLC.

MATERIALS AND METHODS

Reagents—MCDB-104 medium was purchased from Kyokuto Pharmaceutical Industries, Tokyo. Fetal bovine serum (FBS) was purchased from Wako Industries, Tokyo. Fibroblast growth factor (FGF) was extracted from bovine brains by the method of Lobb and Fett (*21*). Manoalide, isolated from the marine sponge *Luffariella variabilis*, was purchased from Wako Industries. 3-(4-Octadecyl)benzoylacrylic acid (OBAA), U73122, U73343, and compound 48/80 were purchased from Funakoshi, Tokyo. Oleyloxyethyl phosphorylcholine (OOPC) was purchased from Cayman Chemical. Radiolabeled arachidonic acid [5,6,8,9,11,12,14,15-^{3}H-(N)]; (specific activity, 3.7 TBq/mmol) and *myo*-[2-^{3}H]inositol (specific activity, 0.78 TBq/mmol) were purchased from Du Pont-New England Nuclear Inc. All other reagents were of ultrapure grade.

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Abbreviations: HUVEC, human umbilical endothelial cells; OBAA, (4-octadecyl)benzoylacrylic acid; OOPC, oleyloxyethylphosphorylcholine; PLA_2, phospholipase A_2; PLC, phospholipase C.

Cell Culture—Human umbilical vein endothelial cells (HUVEC) were obtained by the method of Jaffe *et al.* (*22*). The cells were cultured in gelatin-coated plastic dishes in MCDB-104 medium, that was supplemented with 10% FBS and 70 ng/ml FGF (as well as 100 μg/ml heparin) at 37°C in 5% CO_2 and 95% air. Throughout our experiments, we used cells with a population doubling level of 15-25.

Assay for Viability—Cells were grown until cultures reached sub-confluence and then the medium was replaced with unmodified MCDB-104 medium after one wash with the same medium. Under sub-confluent conditions, the cells barely respond to the removal of growth factors. The cells were then incubated with manoalide, OBAA, OOPC, U73122, U73343, or compound 48/80, or without any of these compounds. Trypsinized cells were counted with a Coulter counter after 16 h. Cells that had detached from the dishes were washed away before the treatment with trypsin. The cells that remained attached to dishes after washing the resultant blebs off were not stained by erythrosine B (5 mg/ml) and were thus regarded as living cells.

Analysis of DNA Fragmentation—Cells (2×10^6) were incubated in a digestion buffer that contained 0.2 mg/ml proteinase K at 50°C for 5 h. The cellular DNA was extracted once with phenol and once with a mixture of phenol, chloroform and 3-methyl-1-butanol (25 : 24 : 1, v/v). After digestion by RNase (final concentration, 0.6 mg/ml) at 37°C for 30 min, the samples were subjected to electrophoresis on a 2% agarose gel in Tris-acetate buffer. The gel was then stained with ethidium bromide and photographed on a UV transilluminator.

Labeling of HUVEC with [^{3}H]Arachidonic Acid—Subconfluent cultures in 34.6-mm (diameter) dishes were incubated with 17.5 MBq of [^{3}H]arachidonic acid for 24 h at 37°C (in MCDB-104 medium plus 10% serum and 70 ng/ml FGF). At the end of the incubation, the medium was sampled for measurement of the remaining radioactivity and the cells were washed three times with MCDB-104 medium without FGF and serum to remove all unincorporated [^{3}H]arachidonic acid. Approximately 80% of the added [^{3}H]arachidonic acid was incorporated by cells during the incubation. The washed cells were harvested, and lipids were extracted from these cells by the method of Sa and Fox (*23*). To ensure that [^{3}H]arachidonic acid had been incorporated at the *sn*-2 position of phospholipids, fatty acids obtained after or without treatment with PLA_2 from snake venom were separated by thin-layer chromatography on Silica Gel G plates (Merck, Germany) with a mixture of hexane, diethyl ether and glacial acetic acid (80 : 20 : 1, v/v) as the mobile phase. Arachidonic acid was detected with I_2 vapor, and radioactivity was determined in a liquid scintillation spectrometer (Aloka LSC732) with an external standard.

Analysis of the Release of [^{3}H]Arachidonic Acid from HUVEC—This experiment was performed according to the published method (*32*). For measurement of the release of [^{3}H]arachidonic acid, prelabeled cells were incubated in

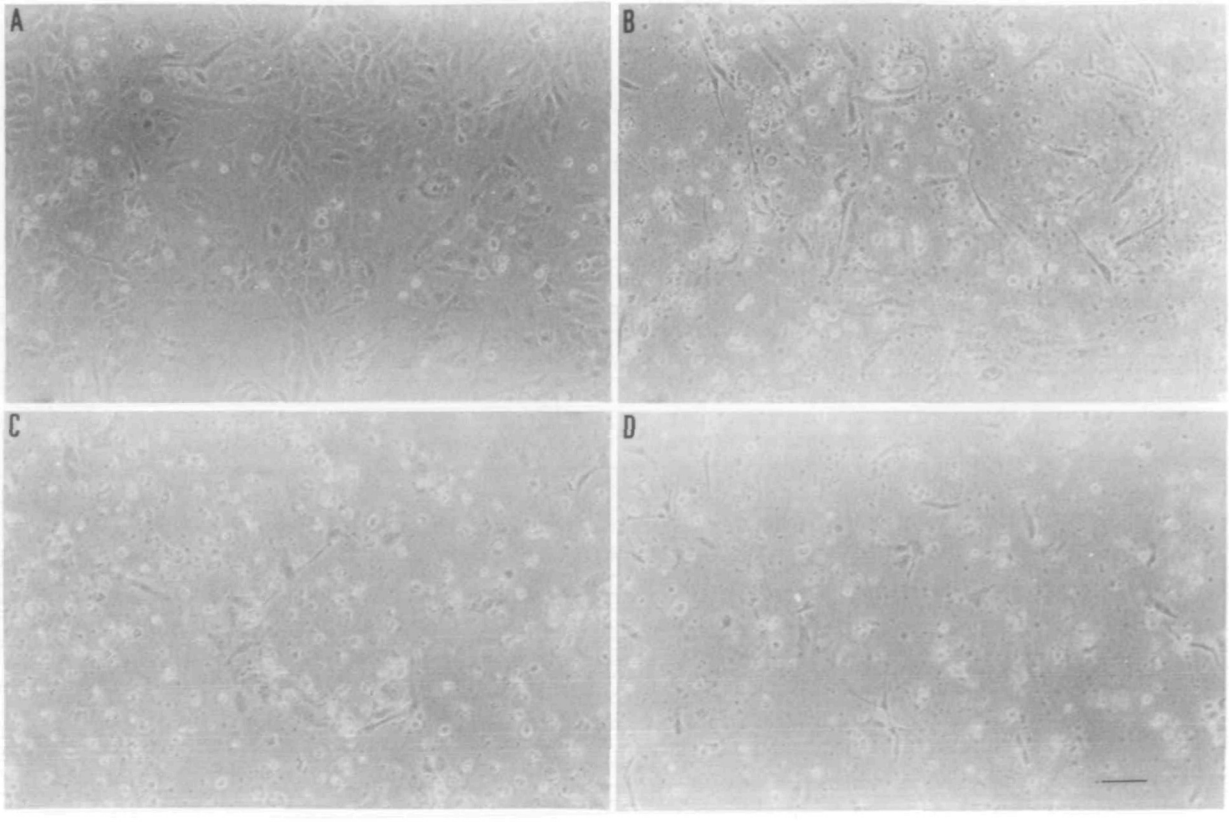

Fig. 1. **Modulation of apoptotic cell death by manoalide, OBAA, and OOPC and associated changes in cell morphology.** (A) Cells 16 h after removal of FGF and serum. (B), (C), and (D) Cells 16 h after the start of treatment with 7 μM manoalide, 5.7 μM OBAA, and 7.5 μM OOPC, respectively, in the absence of FGF and serum. Almost all of the cells treated with these inhibitors of PLA_2 disintegrated into apoptotic bodies. Bar = 100 μm.

MCDB-104 medium with FGF and serum (10% serum and 70 ng/ml FGF) or with an inhibitor of PLA_2 in the absence of FGF and serum, or with 1% serum and 7 ng/ml FGF at 37°C for 5 h. Then the medium was removed and centrifuged to remove cells, and an aliquot of the supernatant was monitored for radioactivity due to [^{3}H]arachidonic acid in the liquid scintillation spectrometer. The radioactive material that was contained in the supernatant mainly comigrated with authentic arachidonic acid when it was separated on a TLC plate.

Assay of the Release of [^3H]Inositol from HUVEC—We

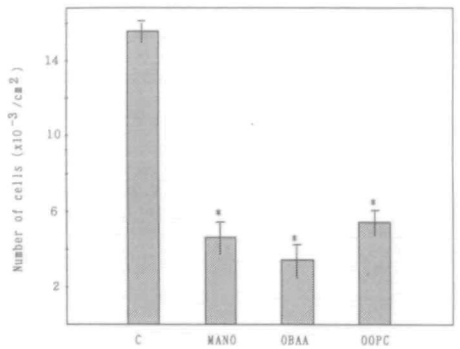

Fig. 2. **Promotion of apoptosis of HUVEC by manoalide, OBAA, and OOPC (7, 5.7, and 7.5 μM, respectively).** The cells that remained on dishes were counted 16 h after the start of treatment. Each value is the mean and SE (bars) of the results of three independent experiments. *$p<0.001$, when compared with the control value.

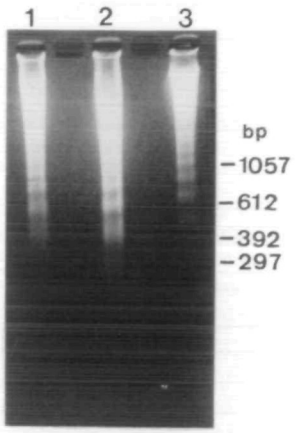

Fig. 3. **Effects of inhibitors of PLA_2 on DNA fragmentation in apoptotic HUVEC that had been deprived of FGF and serum.** DNA was obtained from the entire culture (cells+medium) of each sample at 16 h after the start of treatment. Lanes 1-3, DNA from cells and culture medium together after treatment with 7 μM MANO, 5.7 μM OBAA, and 7 μM OOPC, respectively.

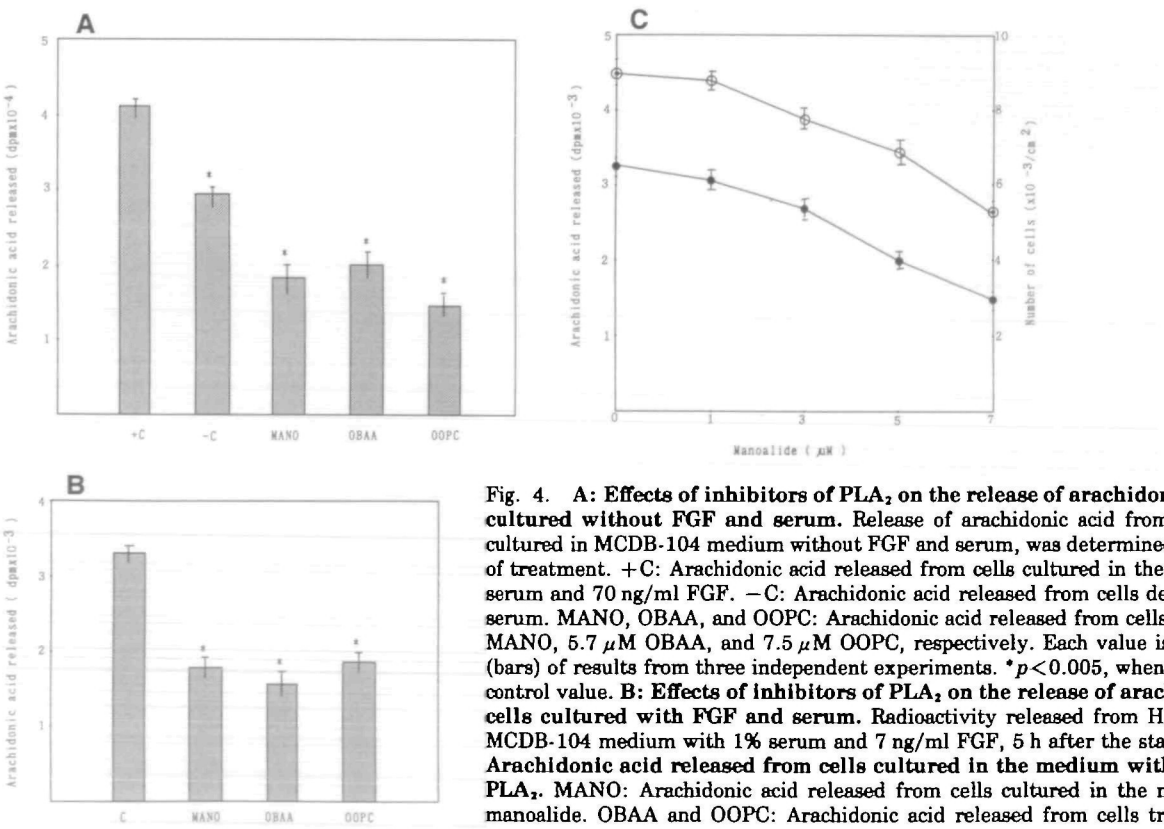

Fig. 4. **A: Effects of inhibitors of PLA_2 on the release of arachidonic acid from cells cultured without FGF and serum.** Release of arachidonic acid from cells, which were cultured in MCDB-104 medium without FGF and serum, was determined 5 h after the start of treatment. +C: Arachidonic acid released from cells cultured in the medium with 10% serum and 70 ng/ml FGF. −C: Arachidonic acid released from cells deprived of FGF and serum. MANO, OBAA, and OOPC: Arachidonic acid released from cells treated with 7 μM MANO, 5.7 μM OBAA, and 7.5 μM OOPC, respectively. Each value is the mean and SE (bars) of results from three independent experiments. *$p<0.005$, when compared with the control value. **B: Effects of inhibitors of PLA_2 on the release of arachidonic acid from cells cultured with FGF and serum.** Radioactivity released from HUVEC, cultured in MCDB-104 medium with 1% serum and 7 ng/ml FGF, 5 h after the start of treatment. **C: Arachidonic acid released from cells cultured in the medium without inhibitors of PLA_2.** MANO: Arachidonic acid released from cells cultured in the medium with 7 μM manoalide. OBAA and OOPC: Arachidonic acid released from cells treated with 5.7 μM OBAA and 7.5 μM OOPC respectively. *$p<0.05$, when compared with the control value. **C: on HUVEC apoptosis.** The release of arachidonic acid from cells cultured in the absence or presence of manoalide at the concentration shown in this figure was measured after 5 h of treatment (filled circle) as described in "MATERIALS AND METHODS." At the same time, the cells that remained on the dishes were counted (open circle). Means and SD were from three independent experiments.

measured the released inositol by the method described in Ref. *33*. Cells were grown in six-well plates. One day before subconfluence, 37 kBq of *myo*-[2-^{3}H]inositol was added in 1 ml of fresh medium to each well. Then, 24 h later, the cells were washed three times with MCDB medium and 5 ml of MCDB medium with inhibitor or without inhibitor was added. The amount of inositol released into the supernatant was determined by scintillation counting.

RESULTS

Effects of Inhibitors of PLA_2 on Apoptosis of HUVEC— When HUVEC were exposed to OBAA at 5.7 μM in the absence of FGF and serum, the cells started to die within 5 h after the addition of OBAA. The morphological changes in cells, observed under the light microscope, were characteristic of apoptotic cell death (Fig. 1C). After 16 h, few cells attached onto the dishes (Fig. 2) while many control cells were still attached to the dishes in the absence of growth factors. When 7 μM manoalide or 7.5 μM OOPC was added to a subconfluent culture of HUVEC, the cells gradually died from 8 h after the addition of either inhibitor of PLA_2, and almost all of the cells disintegrated into apoptotic bodies (Fig. 1, B and D, and Fig. 2). We collected cells after 16 h of such treatment by centrifugation and extracted the DNA. Electrophoretic fractionation of the DNA on an agarose gel confirmed that the death of HUVEC induced by manoalide, OBAA or OOPC was apoptotic cell death (Fig. 3). These inhibitors had no apoptosis-inducing effect in the presence of FGF and serum (data not shown).

To characterize the mechanism by which these inhibitors of PLA_2 induce apoptosis of HUVEC, we monitored the release of arachidonic acid from the cells treated with manoalide, OBAA or OOPC. These three inhibitors of PLA_2 clearly inhibited the release of arachidonic acid from the treated cells at the concentrations at which they induced apoptotic cell death (Fig. 4A). The effect of manoalide on apoptosis of HUVEC as well as on release of arachidonic acid was dose-dependent (Fig. 4C) and all other inhibitors used in this paper showed similar dose-dependencies between the concentration of 0 and 10 μM (data not shown). When we lowered the concentrations of FGF and serum in the medium, these inhibitors could also suppress the release of arachidonic acid (Fig. 4B), although they could not induce apoptosis in the presence of FGF and serum of normal concentration (FGF of 70 ng/ml and serum of 10%).

Effects of Inhibitors of PLC on Apoptosis of HUVEC— To determine whether PLC might be implicated in apoptosis of HUVEC, we exposed subconfluent cultures of cells to 10 μM U73122, an inhibitor of PLC, in the absence of FGF and serum; the cells started to detach from the dish 2 h after the addition of U73122 and then gradually fragmented into apoptotic bodies. After 16 h of treatment, about 70% of the cells had become detached from the dishes (Fig. 6). After 24 h, few cells remained intact (Figs. 5C and 6). In further experiments, in which U73343 (a similar amino-

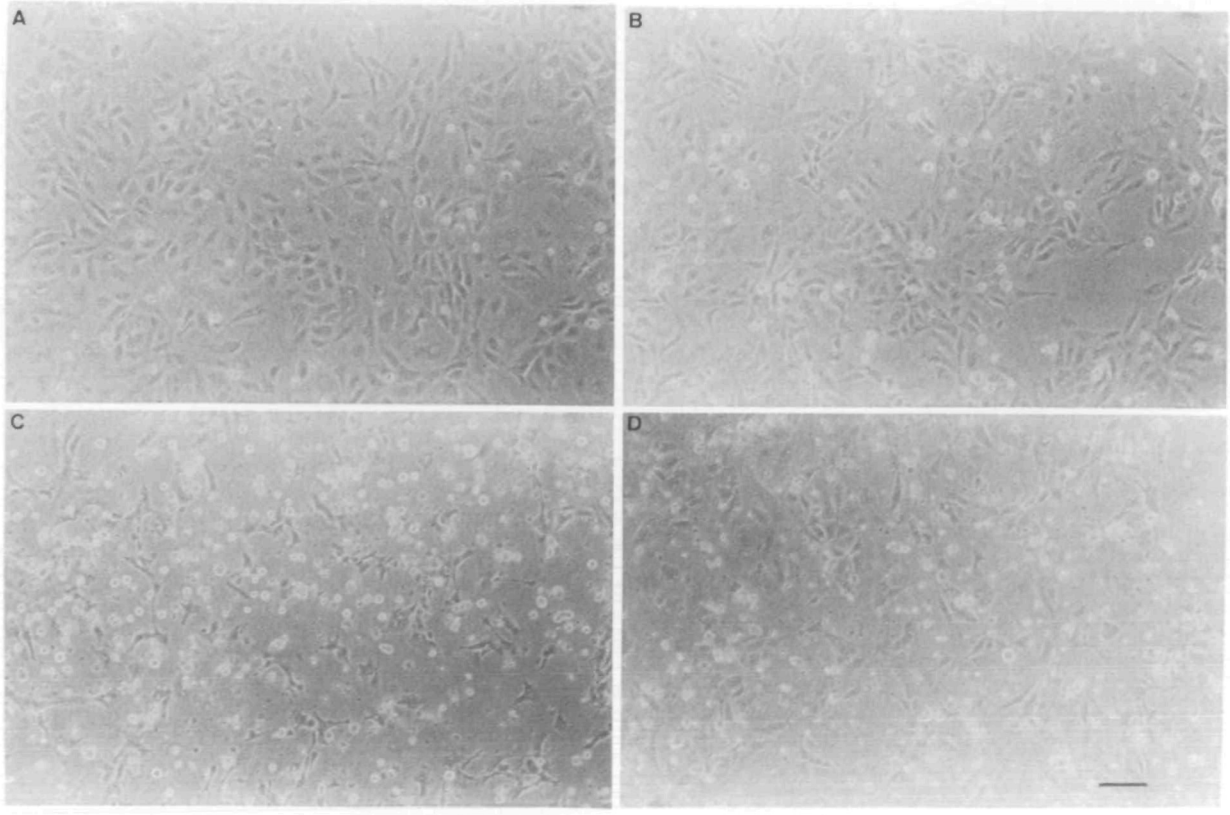

Fig. 5. **Promotion of apoptosis of HUVEC by inhibitors of PLC and associated changes in morphology.** (A) Cells 16 h after deprivation of FGF and serum. (B), (C) and (D) Cells 16 h after the start of treatment with 10 μM U73343, 10 μM U73122, and 30 μg/ml compound 48/80, respectively, in the absence of FGF and serum. Few cells remained intact in cultures that had been treated with inhibitors of PLC. By contrast, cells treated with U73343 were nearly identical to control cells. Bar = 100 μm.

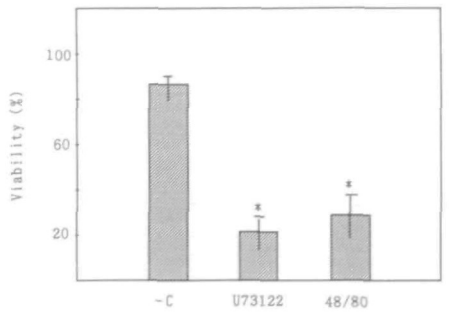

Fig. 6. **Effects of inhibitors of PLC on the viability of HUVEC.** Cells that remained on dishes were counted 24 h after the start of treatment. Cells were treated with 30 μg/ml compound 48/80, 10 μM U73122, or without either drug (−C). Each value is the mean and SE (bars) of results from three independent experiments. $^*p<0.001$, when compared with the control value.

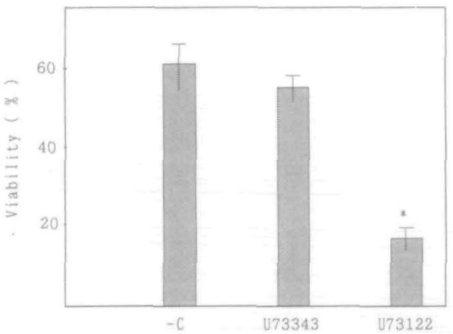

Fig. 7. **Effects of U73343 and U73122 on apoptosis.** The viability of cells cultured in MCDB medium with 10 μM U73122 or 10 μM U73343, 24 h after the start of treatment. −C: The viability of control cells (in the absence of FGF and serum), 24 h after the start of treatment. Each value is the mean and SE (bars) of results from three independent experiments. $^*p<0.005$, when compared with the control vaule.

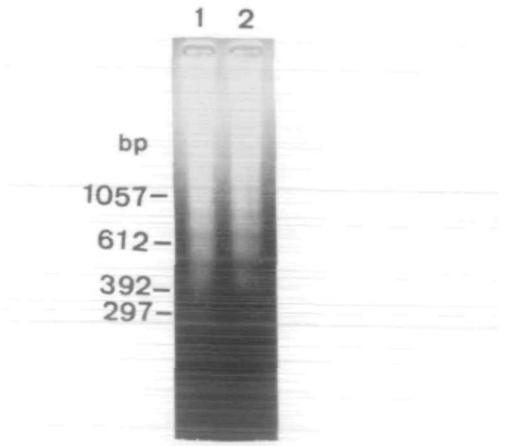

Fig. 8. **Evidence for DNA fragmentation in the presence of inhibitors of PLC.** HUVEC were deprived of FGF and serum and cultured in the presence of U73112 or compound 48/80. DNA was extracted from entire cultures 12 h after the start of treatment. Lane 1, DNA from cells cultured in the medium with 10 μM U73122; lane 2, DNA obtained from cells treated with 30 μg/ml compound 48/80.

steroid that does not inactivate PLC) was used instead of U73122, the viability of cells exposed to U73343 was nearly identical to that of cells under control conditions (Fig. 7).

Compound 48/80 is another inhibitor of PLC. When we exposed cells to this inhibitor at 30 μg/ml in the absence of FGF and serum, the viability was almost the same as that of cells in control cultures after 6 h of treatment. However, after 12 h, the fragmentation of cells that were treated with compound 48/80 was much greater than that of control cells. After 24 h, almost all of the treated cells formed apoptotic bodies (Figs. 5D and 6). These inhibitors of PLC had no apoptosis-inducing effect in the presence of FGF and serum (data not shown).

We collected all of the cells that had been treated with U73122 or compound 48/80 12 h after the start of treatment, and extracted the DNA. Electrophoretic fractionation of the DNA on an agarose gel revealed the fragmentation of the DNA that is characteristic of apoptotic cell death (Fig. 8). Furthermore, we monitored the release of inositol from cells that had been treated with each of these two inhibitors of PLC and from control cells. The amount of inositol released from cells cultured in the medium without FGF and serum gradually increased with time (Fig. 9a). When cells were cultured in the medium with 10 μM U73122 or 30 μg/ml compound 48/80 in the absence of

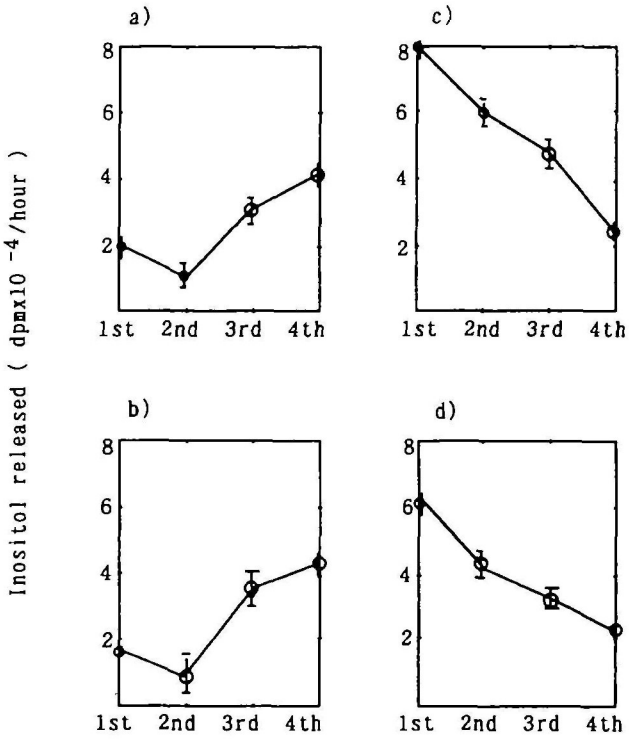

Fig. 9. **Effects of inhibitors of PLC on the release of inositol from HUVEC.** The release of inositol was measured as described in "MATERIALS AND METHODS." (a) Inositol released (per hour) from cells cultured in MCDB medium without FGF and serum. (b), (c), and (d) Inositol released (per hour) from cells treated with 10 μM U73343, 10 μM U73122, or 30 μg/ml compound 48/80, respectively, in the absence of FGF and serum. The release of inositol from cells treated with the two inhibitors was blocked. Each value is the mean and SE (bars) of results from three independent experiments.

FGF and serum, the amount of inositol released from these cells gradually declined with time (Fig. 9, c and d). By contrast, in the presence of U73343, the amount of released inositol increased with time, as it did in the control (Fig. 9b).

DISCUSSION

Apoptosis of HUVEC can be induced by deprivation of FGF and serum, and protein kinase C has been implicated in the signaling pathway that leads to such apoptosis (*6, 7*). In this study, to understand whether PLA_2 and PLC might be implicated in HUVEC apoptotic signal transduction, we first obtained evidence that PLA_2 and PLC might function in HUVEC apoptosis induced by deprivation of FGF and serum (Figs. 4 and 9) (*30*), then we confirmed this by using the inhibitors of PLA_2 and PLC. Our results suggest that PLA_2 and *PLC* and, simultaneously, the release of arachidonic acid and inositol from these cells might be involved in HUVEC apoptotic signal transduction.

Many experiments have shown that PLA_2 is involved in the intracellular signaling networks of protein kinase C and G-proteins (*24*) and, moreover, the release of arachidonic acid, which is mainly catalyzed by phospholipase A_2, has an important role in the regulation of cell proliferation (*11*). Other experiments have shown that stimulation of mitogenesis by growth factors is accompanied by marked changes in the rate of release and metabolism of arachidonic acid (*25-28*).

The present study showed that manoalide, OBAA and OOPC, three inhibitors of PLA_2, promoted apoptosis of HUVEC in culture and, in addition, that the release of arachidonic acid from the cells treated with these inhibitors of PLA_2 declined with the progression of apoptosis. On the other hand, a putative $cPLA_2$-specific inhibitor, the trifluoromethyl ketone analogue of arachidonic acid (*31*) showed no apoptotic effect although the reagent induced necrotic cell death at high concentration (data not shown). Recent experiments have shown that the release of arachidonic acid from membrane phospholipids is mainly catalyzed by PLA_2. Two forms of PLA_2s, known as secreted PLA_2 ($sPLA_2$) and cytosolic PLA_2 ($cPLA_2$), seem to be involved in this regulation, depending on the cell type and on the stimulus (*38*). Our results indicate that $sPLA_2$ might be mainly implicated in the signaling pathway to apoptosis induced by deprivation of FGF and serum in HUVEC and that the arachidonic acid cascade might play an important role in this apoptotic signal-transduction system. The experiment to see whether the effect of PLA_2-inhibitors could be overcome by the addition of a specific product of PLA_2 action was not successful. Simple addition of such a product to the culture medium of cells had no effect (data not shown). In cell biology, the phenomenon that exogenous and endogenous factors do not have the same functions is common (*33, 34*), and our result provides another example.

PLC is another critical enzyme in the intracellular signaling network (*14-16*). Both phosphatidylinositol-specific PLC and phosphatidylcholine-specific PLC are found in cells, and both have been implicated in cell proliferation (*15, 16*). Phosphatidylinositol-specific PLC mediates the effects of growth factors and oncogenes on cell proliferation (*17-20*), while inositol lipids have important roles in cellular phospholipid signaling (*10, 29*). Some experiments showed that phosphatidylcholine-specific PLC might be involved in the Fas/APO-1-generated apoptotic signal in lymphoid cells (*35*), but little is known about the role of phosphatidylinositol-specific PLC in apoptosis. The two inhibitors of PLC, U73122 and compound 48/80, used in this study are both phosphatidylinositol-specific. Each clearly promoted the apoptosis of HUVEC in culture, simultaneously inhibiting the release of inositol from these cells. As we reported in a previous paper, alkyllysophospholipid induces the apoptotic cell death of endothelial cells (*30*). This agent is also known to be a specific inhibitor of the activity of PLC (*16*). These results together suggest strongly that phosphatidylinositol-specific PLC and inositol metabolism are involved in the signaling pathway to apoptosis of HUVEC.

In this study, we also examined the effects of these inhibitors of PLA_2 and PLC on HUVEC in the presence of FGF and serum, and found that they could not induce apoptosis under this condition. It has been shown that the balance of apoptosis-inducing and apoptosis-inhibiting signals in the regulation of cell functions is very important, and when this balance is damaged the functions of cells will be disordered (*36*). One view of apoptosis is that it is a cellular response to conflicting signals (*37*). The result that in the presence of FGF and serum, the inhibitors of PLA_2 and PLC failed to promote apoptosis of HUVEC provides some support for this notion.

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