Sustained JNK activation induces endothelial apoptosis: studies with colchicine and shear stress

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Hu, Ying-Li, Song Li, John Y.-J. Shyy, and Shu Chien. Sustained JNK activation induces endothelial apoptosis: studies with colchicine and shear stress. *Am. J. Physiol.* 277 (*Heart Circ. Physiol.* 46): H1593–H1599, 1999.—The disruption of microtubules by treating bovine aortic endothelial cells with 10^{-7}–10^{-5} M colchicine caused apoptosis, as evidenced by DNA laddering and TdT-mediated dUTP nick end labeling fluorescence staining. Colchicine treatment also induced a sustained activation of c-Jun NH_2-terminal kinase (JNK) that lasted for ≥12 h. The blockade of JNK activity by using the negative interfering mutant JNK(K-R) markedly decreased the apoptosis induced by colchicine. Exposure of bovine aortic endothelial cells to laminar shear stress (12 dyn/cm²) caused a transient (<2 h) activation of JNK, and there was no induction of apoptosis. The sustained activation of JNK may play a significant role in the apoptosis induced by colchicine.

cell death; endothelial cells

VASCULAR ENDOTHELIAL CELLS (ECs), which form the inner lining of the blood vessel wall, play an important role in the regulation of vascular homeostasis. The cytoskeletal system, including actin microfilaments, intermediate filaments, and microtubules (MTs), is essential in EC adhesion, migration, and mitosis. The MTs, which emanate from the MT organization center near the nucleus, interact with actin microfilaments to coordinate EC structural organization and participate in intracellular signal transduction.

The processes of cell survival and cell apoptosis involve highly regulated signaling pathways. Apoptosis, or programmed cell death, is characterized by morphological alterations that include cell shrinkage, membrane blebbing, chromosome condensation, and DNA fragmentation (7). Colchicine, an inhibitor of MT assembly that causes MT disruption, has been found to trigger apoptosis in primary neural cell cultures (2), colonic crypt cells (13), interphase lymphocytes (4), HL-60 cells (20), chronic lymphocytic leukemia cells (1), and rat hepatocytes (23). Apoptosis can also be induced by gamma irradiation (8) and ultraviolet (UV) light (5) in mammalian cells, and the c-Jun NH_2-terminal kinase (JNK) is activated in these apoptotic cells.

Laminar shear stress causes ECs to elongate and align with flow under in vivo and in vitro conditions (9). These morphological changes are accompanied by alterations in the cytoskeleton, e.g., the formation of actin stress fibers (6, 18, 25), the axial realignment of MTs, and the transient repositioning of the MT organization center (6, 11, 12). The cytoskeletal reorganization in response to laminar shear stress has been linked to gene expression (14, 19), and MT disruption has been shown to block the shear stress-induced EC morphological change and actin stress fiber formation (18). Laminar shear stress can also activate the Ras-mitogen-activated protein kinase kinase (MEKK)-JNK pathway in ECs in a transient manner (17).

Thus, although gamma irradiation, UV light, and shear stress cause JNK activation, their effects on cell fate (apoptosis or survival) are not the same. The present study was designed to test the hypothesis that the temporal nature of JNK activation is important in determining cell fate by comparing the effects of colchicine and shear stress. Our results indicate that a sustained JNK activation is a critical step in the colchicine-induced apoptosis and that the transient JNK activation by shear stress does not lead to apoptosis.

MATERIALS AND METHODS

Cell culture. Bovine aortic ECs (BAECs) were isolated from bovine aorta by using collagenase or by gentle scraping with a rubber policeman. The BAECs were cultured in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FCS (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, and penicillin-streptomycin and sodium pyruvate at 1 mM each. The cells were grown on glass slides (3 × 1 in.) or dishes precoated with 2% gelatin (Sigma Chemical, St. Louis, MO) or in DMEM containing 10% FCS. The cells were maintained in a humidified 95% air-5% CO_2 incubator at 37°C.

Cell treatment. The stock solution of colchicine (1 mM; Sigma Chemical) was dissolved in absolute ethanol. BAECs in culture medium described above were treated with colchicine or with ethanol as a vehicle control. Paired experiments were conducted on confluent BAECs. The experimental groups were subjected to colchicine treatment with final concentrations of 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M or shear stress at 12 dyn/cm². The control groups were kept under a static condition without colchicine treatment.

Shear stress experiment. The shear stress experiments were conducted in a rectangular flow chamber. The chamber, a reservoir, and a circulation circuit were filled with fresh DMEM. The medium was driven by using a flow loop under a constant-pressure head, such that the BAECs were subjected to a laminar shear stress at 12 dyn/cm². A roller pump was used to return the chamber outflow to the feed reservoir. The medium was kept at a constant temperature of 37°C and equilibrated with a gas mixture of 95% air-5% CO_2. In the static control group the BAECs were kept in the chamber for the same duration under the same conditions but without flow.

Immunofluorescence staining for MTs. After the experimental period the BAECs were fixed with 1% formaldehyde for 20

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min at 37°C in PBS. The fixed cells were permeabilized, and nonspecific binding was blocked by preincubation with PBS containing 0.3% Triton X-100 and 1% normal goat serum. To visualize MTs, the cells were incubated with an anti-α-tubulin monoclonal antibody (MAb; Sigma Chemical) in PBS containing 0.3% Triton X-100, then with a tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibody. For negative controls, normal goat serum was used, and this was followed by the anti-α-tubulin MAb without the secondary antibody or the TRITC-labeled secondary antibody without the anti-α-tubulin MAb. The slides were mounted with the SlowFade antifade reagent (Molecular Probes, Eugene, OR) and viewed under a Nikon microscope (Microphot-FX). The rhodamine fluorescence was studied with an excitation filter at 546 nm, a dichroic mirror at 580 nm, and a barrier filter at 580 nm.

DNA plasmids and transfection. RasN17, MEKK(K-M), JNK(K-R), and hemagglutinin epitope-tagged JNK1 (HA-JNK1) plasmids were described previously (17). RasN17 is a dominant negative mutant of Ras, MEKK(K-M) is a catalytically inactive mutant of MEKK, and JNK(K-R) is a negative mutant of JNK1. HA-JNK1 was used as an exogenous JNK1 without mutation. In control groups the cells were transfected with the empty pSRα cloning vector. The various DNA plasmids were transfected into BAECs at 80% confluence by using the lipofectamine method (Life Technologies). The pSV-β-galactosidase plasmid (pSV-β-gal), which contains a β-galactosidase gene driven by simian virus 40 promoter and enhancer, was cotransfected for the monitoring of transfection efficiency. After incubation for 6 h, the cells were washed with DMEM and incubated with fresh DMEM for another 24–48 h to reach confluence. The cells in the tissue culture flasks were then seeded on glass slides for use in the colchicine or shear stress experiments.

JNK activity assay. To assay the endogenous JNK activity, BAECs were lysed in a kinase lysis buffer, which contained 25 mM HEPES, pH 7.4, 0.5 M NaCl, 1% Triton X-100, 0.1% deoxycholate, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 50 mM NaF, 10 mM Na_3VO_4, and 2 mM β-glycerophosphate. JNK was immunoprecipitated with an anti-JNK1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and protein A-Sepharose beads. To perform immunocomplex kinase assays, the immunoprecipitates were washed twice in the lysis buffer and twice in a kinase assay buffer (25 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 20 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM dithiothreitol). Two micrograms of glutathione *S*-transferase (GST)-c-Jun-(1–79) fusion protein and 10 µCi of [γ-^{32}P]ATP (ICN, Irvine, CA) in 30 µl of a kinase assay buffer containing 25 mM ATP were added to each immunocomplex pellet for kinase reaction at 30°C for 20 min. The phosphoproteins were separated by SDS-PAGE, and the gels were dried for autoradiography. To assess the activation of the exogenous HA-JNK1 that had been transfected into BAECs, a mouse anti-HA MAb (Boehringer Mannheim, Indianapolis, IN) was used to immunoprecipitate HA-JNK1 for the determination of its kinase activity by using GST-c-Jun-(1–79) as the substrate.

DNA fragmentation. DNA fragmentation was detected by gel electrophoresis according to the procedure described by Wyllie et al. (26). Cells were pelleted at 400 *g* and washed twice with ice-cold Tris-buffered saline (137 mM NaCl, 2.7 mM KCl, 25 mM Tris, pH 7). The pellets were resuspended in 50 µl of Tris-EDTA (1 mM EDTA, 10 mM Tris, pH 8.0) and lysed with 0.5 ml of an extraction buffer (0.1 M EDTA, 0.5% SDS, 10 mM Tris, pH 8.0) containing 0.5 mg/ml proteinase K. After overnight incubation at 50°C, DNA was extracted from the samples and ethanol precipitated for agarose gel electrophoresis. The DNA separated in the gel was visualized by UV fluorescence.

Triple-staining immunofluorescence for apoptotic cells. Apoptotic cells were detected by using the TdT-mediated dUTP nick end labeling (TUNEL) system (Promega, Madison, WI). BAECs transfected with HA-JNK1, the negative mutant JNK(K-R), or the empty cloning vector (pSRα) were studied. All cells were cotransfected with pSV-β-gal for identifying the transfected cells. The cotransfection efficiency was between 10 and 20%. The confluent BAEC monolayers were washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. After permeabilization with 0.2% Triton X-100 in PBS, the specimens were stained with the TUNEL reaction mixture containing fluorescein-labeled nucleotides, and the reaction was terminated by adding 2× saline-sodium citrate. After TUNEL staining the specimens were subjected to immunostaining with a mouse anti-β-gal MAb (Santa Cruz Biotechnology), then with the anti-mouse TRITC-conjugated secondary antibody for the identification of the transfected cells. After the cells were washed with PBS, the nuclear chromatin was detected by staining with PBS containing 8 µg/ml of the DNA-binding fluorochrome bisbenzimide trihydrochloride (Hoechst-33258, Boehringer Mannheim) for 15 min. The specimens were washed, and the green fluorescence of fluorescein-12-dUTP was detected in the fluorescence microscope with an excitation filter at 495 nm and an emission filter at 520 nm to identify apoptotic cells. The red fluorescence of rhodamine was detected with an excitation filter at 546 nm and an emission filter at 580 nm. The blue fluorescence of bisbenzimide was detected with an excitation filter at 365 nm and an emission filter at 400 nm. In a given field of the slide under the fluorescence microscope, the same group of cells was examined three time by changing filters to view blue, red, and green fluorescence, which represented cell nuclei, transfected cells, and apoptotic cells, respectively, in this triple-staining technique. The images were recorded on film, transferred to a Power Macintosh 8100/80 computer, and analyzed by using the Adobe Photoshop (Adobe System, Mountain View, CA).

RESULTS

Effect of colchicine and shear stress on JNK activity. We first investigated the effect of 10^{-6} M colchicine on the JNK kinase activity of BAEC monolayers as a function of time. The JNK activity, as indicated by the phosphorylation of its substrate GST-c-Jun-(1–79), showed a significant increase after 0.5 h of colchicine treatment, a greater effect at 1 h, and a maximum plateau at 6 and 12 h (Fig. 1*A*). Compared with the untreated cells, densitometric analysis indicated that the JNK activities increased 2.7-, 3.6-, 5.0-, and 4.9-fold in cells treated with colchicine for 0.5, 1, 6, and 12 h, respectively. We then examined the dose-response relationship of the colchicine-induced activation of JNK. BAEC monolayers were treated with 10^{-8}–10^{-5} M colchicine, then subjected to JNK activity assays. Colchicine at 10^{-6} and 10^{-5} M caused a sustained activation of JNK (Fig. 2). The degree of JNK activation was less at 10^{-7} M colchicine than at 10^{-5} or 10^{-6} M, and the onset was later. At 10^{-8} M, colchicine had no detectable effect on JNK activity.

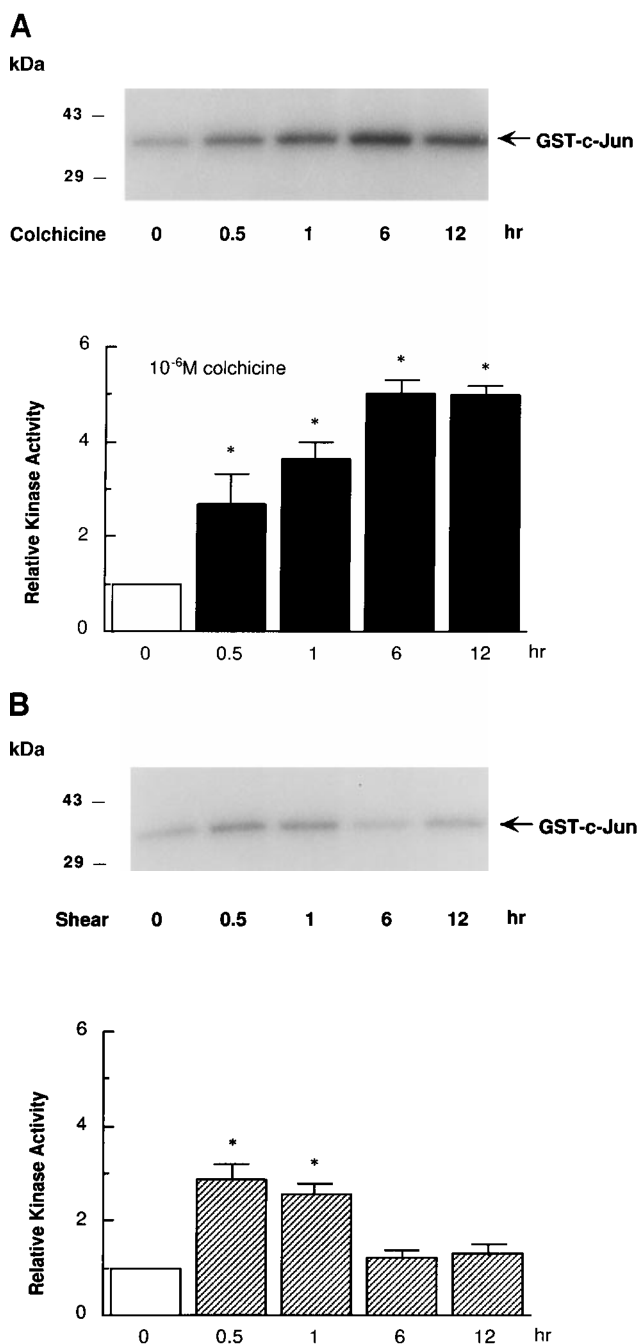

Fig. 1. Responses of c-Jun NH_2-terminal kinase (JNK) to colchicine and laminar shear stress. *A*: disruption of endothelial cell (EC) microtubules by colchicine causes a sustained activation of JNK. Monolayers of bovine aortic ECs (BAECs) were treated with 10^{-6} M colchicine for various durations. JNK activity was assessed by immunocomplex kinase activity assay with glutathione *S*-transferase (GST)-c-Jun as substrate. Amount of phosphorylated GST-c-Jun reflects JNK activity. Densitometric analysis of autoradiographs indicates a sustained increase in JNK activity in colchicine-treated BAECs. Values are means ± SD from ≥3 experiments. *Significant difference ($P < 0.05$) between colchicine-treated and control cells. *B*: shear stress causes a transient activation of JNK. Monolayers of BAECs were subjected to shear stress (12 dyn/cm²) for various durations. Densitometric analysis of autoradiographs indicates that JNK activity in BAECs was increased by application of shear stress for 0.5 and 1 h but decreased to control level at 6 and 12 h. Values are means ± SD from ≥3 experiments. *Significant difference ($P < 0.05$) between sheared and static cells.

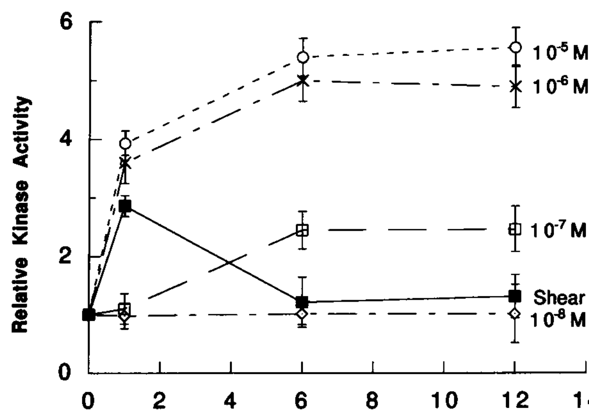

Fig. 2. JNK activation in BAECs by colchicine as a function of dose and time and by laminar shear stress as a function of time. BAECs were treated with 10^{-8}–10^{-5} M colchicine or exposed to shear stress at 12 dyn/cm². Values are means ± SD.

Exposure of BAECs to shear stress (12 dyn/cm²) also led to an activation of JNK (Fig. 1*B*). The JNK activity increased 2.8- and 2.5-fold in cells exposed to shear stress for 0.5 and 1 h, respectively, compared with the static control cells. Continued application of shear stress for 6 and 12 h, however, caused the JNK activity to decrease to the static control level. Thus, in contrast to the sustained activation of JNK by colchicine, laminar shear stress induced a transient activation of JNK. Figure 2 shows a composite plot of JNK activation in response to shear stress and different doses of colchicine. The peak increase in JNK activity induced by shear stress was equal to or higher than the maximum JNK activity after 10^{-7} M colchicine, but the response to JNK was transient in contrast to the sustained response to colchicine.

Immunohistochemical staining with anti-α-tubulin MAb showed the intact structure of MTs in control BAECs (Fig. 3*A*). After 1 h of incubation with 10^{-6} M colchicine, cells showed dissolution of the fine fibrillar pattern of tubulin into diffuse staining (Fig. 3*B*). Shear stress caused the well-known elongation of ECs and orientation of MTs in the direction of flow; there was no MT dissolution (Fig. 3*C*).

Blockade of the Ras-MEKK pathway did not abolish the colchicine activation of JNK. We previously showed that the shear stress activation of JNK is mediated by the Ras-MEKK pathway (17). To investigate whether Ras and MEKK are upstream to the colchicine-activated JNK, we examined the effects of RasN17, a dominant negative mutant of Ras, and MEKK(K-M), a catalytically inactive mutant of MEKK. HA-JNK1 was cotransfected with RasN17, MEKK(K-M), or an empty vector pSRα into BAECs. If the Ras-MEKK pathway is upstream to the colchicine-induced activity of JNK, the use of RasN17 or MEKK(K-M) should attenuate the colchicine-induced JNK kinase activity, as assessed by the phosphorylation of GST-c-Jun-(1–79). As shown in Fig. 4, treatment with 10^{-6} M colchicine for 1 h caused an increase in JNK activity over the untreated control in all three groups. Densitometric analysis showed that colchicine treatment caused 2.2-, 1.8-, and 2.7-fold

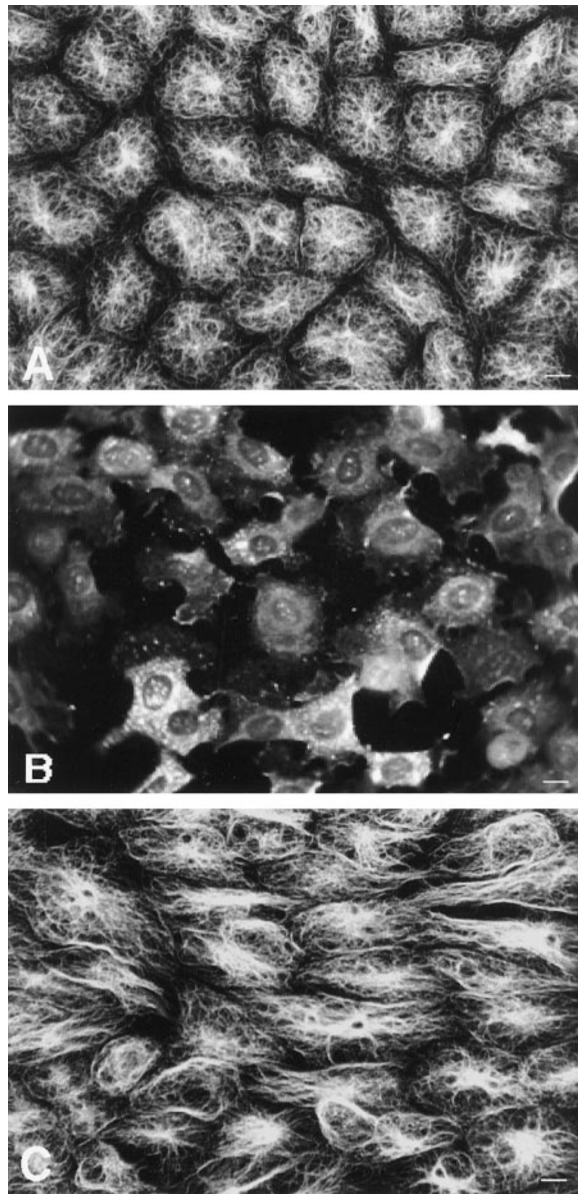

Fig. 3. Immunostaining with anti-α-tubulin in BAECs. *A*: static control cells. *B*: cells exposed to 10^{-6} M colchicine for 1 h, then kept in fresh medium for 11 h. *C*: cells subjected to shear stress at 12 dyn/cm^2 for 12 h. Shear stress resulted in an alignment of microtubules with direction of flow. Colchicine treatment resulted in diffuse α-tubulin staining consistent with microtubule disruption. Antibody stained fiber network and components. Scale bars, 10 μm.

increases of JNK activity in BAECs transfected with pSRα, RasN17, or MEKK(K-M), respectively; there was no statistically significant difference among these groups. Therefore, unlike the shear stress activation of JNK, the colchicine activation of JNK is not mediated by the Ras-MEKK pathway.

Colchicine, but not shear stress, induces endothelial apoptosis. The effect of colchicine on EC apoptosis, as demonstrated by DNA fragmentation, is shown in Fig. 5*A*. Agarose gel electrophoresis of DNA extracted from BAECs that had been incubated with 10^{-7}, 10^{-6}, and 10^{-5} M colchicine for 24 h showed the typical ladder formation of apoptosis; this did not appear in the cells treated at 10^{-8} M. In BAECs exposed to 10^{-6} M colchicine, DNA fragmentation occurred in a time-dependent fashion, being visible after 8 and 24 h, but not at 1 and 4 h (Fig. 5*B*). In concurrent experiments, exposure of BAECs to shear stress (12 dyn/cm^2) for 9 h did not induce apoptosis, as indicated by the lack of DNA fragmentation (Fig. 5*C*).

Role of JNK in the colchicine-induced apoptosis. To test the hypothesis that the induction of apoptosis by colchicine is mediated by JNK activation, BAECs were transfected with JNK(K-R), a dominant negative mutant of JNK, in combination with pSV-β-gal. As controls, BAECs were cotransfected with HA-JNK1 or the empty vector pSRα, together with pSV-β-gal. The transfected cells were subjected to colchicine treatment (10^{-6} M) for 1 h or kept as untreated control. With use of the triple-staining immunofluorescence technique, the cytoplasm of the transfected cells was stained red due to the expression of β-gal. The nuclei appeared blue because of the Hoechst-33258 staining. Apoptosis was identified by TUNEL labeling in the presence of TdT enzyme by the green fluorescence of fluorescein. The fluorescence photomicrographs in Fig. 6 exemplify the results of such a triple-staining study in which BAECs were treated with 10^{-6} M colchicine after transfection with the negative mutant JNK(K-R) (Fig. 6, *A–C*) or HA-

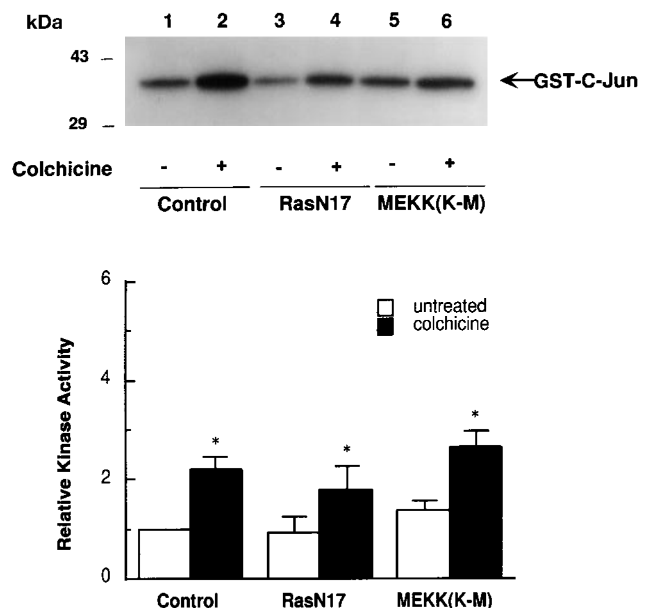

Fig. 4. RasN17 and MEKK(K-M) do not block colchicine-induced JNK activity. BAECs were transiently transfected with empty vector pSRα (*lanes 1* and *2*), RasN17 (*lanes 3* and *4*), or MEKK(K-M) (*lanes 5* and *6*), all in combination with hemagglutinin epitope-tagged JNK1 (HA-JNK1). These plasmid-transfected BAECs were treated with 10^{-6} M colchicine for 1 h (*lanes 2, 4,* and *6*) or kept as untreated controls (*lanes 1, 3,* and *5*). Cells were lysed, and activities of JNK were assayed using procedures described in Fig. 1. Graph shows densitometric analysis of autoradiographs. Values are means ± SD from ≥3 experiments. *Significant increase in JNK activity in all 3 groups of experiments after colchicine treatment ($P < 0.05$). Thus negative mutants RasN17 and MEKK(K-M) did not block increase in JNK activity after colchicine treatment.

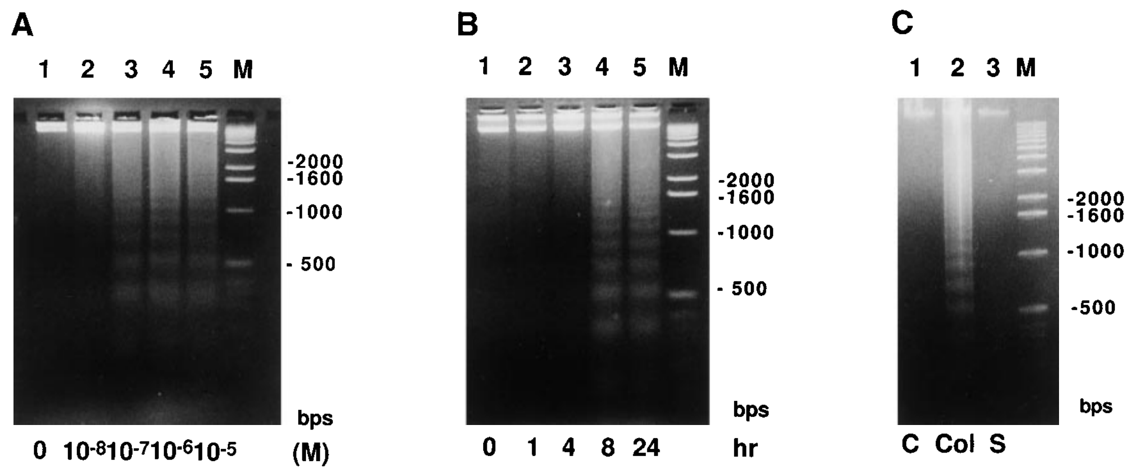

Fig. 5. Colchicine, but not shear stress, induces EC apoptosis. *A*: BAEC monolayers were treated with 10^{-8}–10^{-5} M colchicine for 24 h. DNA was isolated, subjected to agarose gel electrophoresis, and visualized by ethidium bromide staining. Colchicine at $\geq 10^{-7}$ M induced DNA fragmentation in BAECs. *Lane 1*, BAEC control; *lanes 2–5*, BAECs treated with various concentrations of colchicine for 24 h; *lane M*, DNA markers. *B*: BAEC monolayers were treated with 10^{-6} M colchicine for different lengths of time, then subjected to DNA fragmentation assays. BAEC apoptosis was induced with colchicine treatment for ≥8 h. *Lane 1*, BAEC control; *lanes 2, 3, 4,* and *5*, BAECs treated with 10^{-6} M colchicine for 1, 4, 8, and 24 h, respectively; *lane M*, DNA markers. *C*: BAECs were kept as untreated static control (C), treated with colchicine (Col), and subjected to shear stress (S) prior to DNA fragmentation assays. Note absence of apoptosis in BAECs subjected to shear stress. *Lane 1*, BAECs kept as static control; *lane 2*, BAECs treated with 10^{-6} M colchicine for 8 h; *lane 3*, BAECs exposed to shear stress (12 dyn/cm^2) for 8 h; *lane M*, DNA markers.

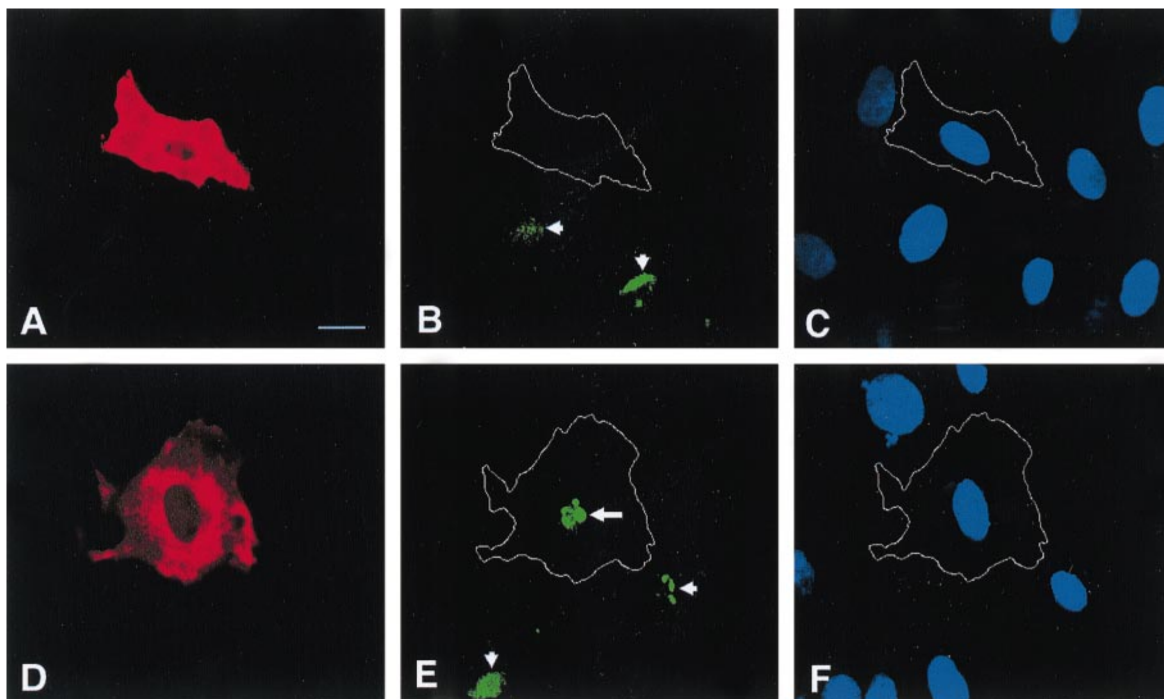

Fig. 6. Triple-fluorescence staining of BAECs to show effect of transfection of negative mutant JNK(K-R) on colchicine-induced apoptosis. *A–C*: photomicrographs from same field of a specimen that had been transfected with negative mutant JNK(K-R) before treatment with 10^{-6} M colchicine. In *A*, β-galactosidase staining demonstrates a successfully transfected cell. In *B*, TdT-mediated dUTP nick end labeling fluorescence staining demonstrates apoptosis in 2 cells (arrowheads), but cell transfected with JNK(K-R) (outline traced from *A*) did not undergo apoptosis. In *C*, Hoechst-33258 staining demonstrates nuclei of 9 cells in field, including transfected cell shown in *A* (outline traced) and 2 apoptotic cells in *B* (arrowheads). *D–F*: photomicrographs from same field of a specimen that had been transfected with HA-JNK1 before treatment with 10^{-6} M colchicine. In *D*, β-galactosidase staining demonstrates a successfully transfected cell. In *E*, TdT-mediated dUTP nick end labeling fluorescence staining demonstrates apoptosis in 3 cells, including 2 untransfected cells (arrowheads) and also cell transfected with HA-JNK1 (arrow on cell outline traced from *D*). In *F*, Hoechst-33258 staining demonstrates nuclei of 8 cells in field, including transfected cell (arrow on cell outline traced from *D*) and 2 apoptotic cells in *E*. Scale bar, 10 μm.

JNK1 (Fig. 6, *D–F*), together with pSV-β-gal and Hoechst-33258. Figure 6, *A* and *D*, shows one successfully transfected cell with positive β-galactosidase staining among the eight to nine cells with nuclei that stained with Hoechst-33258 (Fig. 6, *C* and *F*). Colchicine treatment caused apoptosis in the cell cotransfected with HA-JNK1 (Fig. 6*E*), but not in the cell cotransfected with JNK(K-R) (Fig. 6*B*).

Such triple-staining studies were performed on each of the experimental and the parallel control groups. The percentage of apoptotic cells among the transfected cells was calculated as shown in Fig. 7. For each group the results were obtained from at least 3 independent experiments, with 150 transfected cells counted in each experiment. In cells cotransfected with the empty vector pSRα, the percentage of transfected cells that showed apoptosis was 4.6 ± 0.29% (mean ± SD) without colchicine treatment and increased significantly to 8.4 ± 0.15% after treatment with 10^{-6} M colchicine (Fig. 7). The untransfected cells showed essentially the same results, i.e., 5.2 ± 0.10% apoptotic cells in untreated control and 8.8 ± 0.12% after colchicine (data not shown). In cells cotransfected with HA-JNK1, colchicine treatment caused a significant increase in apoptotic cells from 5.0 ± 0.12 to 12.7 ± 0.24% (Fig. 7). The greater increase than seen in the pSRα-transfected cells may be attributed to the increase in total JNK as a result of the transfection of the exogenous JNK. In cells cotransfected with JNK(K-R), the percentage of apoptotic cells in untreated control was 5.5 ± 0.25%, which was not significantly different from that in the untreated controls of other groups. Colchicine treatment of these JNK(K-R)-transfected cells did not cause any significant increase in apoptosis, being 6.1 ± 0.14% (Fig. 7).

DISCUSSION

Development and homeostasis in multicellular organisms involve programmed cell death as well as cell proliferation (21). Physiological cell death through apoptosis is positively and negatively regulated by many factors, including the aging process and environmental stresses (22). The integrity of the MT-based cytoskeletal structure is critical for cell survival, inasmuch as MT disruption has been shown to induce apoptosis in several cell types (1, 2, 4, 13, 20). In the present study we demonstrated that the MT disruption agent colchicine causes apoptosis in BAECs and that this colchicine-induced apoptosis is dependent on a sustained activation of JNK. The importance of JNK is shown by the blockade of the colchicine-induced apoptosis by the negative mutant JNK(K-R).

The importance of the sustained nature of the JNK activation is shown by parallel investigations on the effects of laminar shear stress. The continued application of shear stress at 12 dyn/cm² for 12 h caused a transient JNK activation with a peak value slightly greater than the maximum value induced by 10^{-7} M colchicine (Fig. 2). However, this transient activation of JNK by shear stress, unlike the sustained activation of JNK by colchicine, was not accompanied by apoptosis. The finding that laminar shear stress causes a transient activation of JNK and protects ECs from being driven into tumor necrosis factor-α-induced apoptosis is in agreement with the concept that transient JNK activation does not lead to apoptosis (10).

The effect of colchicine on JNK activation differs from that of shear stress not only in their relative persistence, but also in their upstream signaling pathway. Cotransfection of RasN17 and MEKK(K-M) into BAECs significantly reduced the shear stress activation of HA-JNK1 (17), but this had no effect on the JNK activation by colchicine (Fig. 4), indicating that the JNK activation by colchicine is mediated by a pathway different from that for shear activation. The nature of the signaling pathway leading to JNK activation by colchicine remains to be elucidated. It has been shown that the signals mediated by death receptors (e.g., Fas receptor) or environmental stress can activate JNK (15). Daxx, interacting with the death domain of Fas receptor, can activate JNK and apoptosis (27). Environmental stresses such as UV light are likely to act directly on the cell membrane to activate acid sphingomyelinase and generate ceramide, thus initiating signals to activate JNK (16, 24).

The concept that a sustained activation of JNK leads to apoptosis is supported by the findings that the apoptosis induced by gamma irradiation and UV light and by anti-Fas treatment is accompanied by a sustained JNK activation. Furthermore, overexpression of activated JNK causes apoptosis, and a negative mutant of JNK1 prevents the apoptosis induced by gamma irradiation and UV light (5).

In the downstream direction, the sustained JNK activation may upregulate c-Jun, which in turn leads to cell death through its transcriptional activity. The c-Jun-mediated apoptosis can be blocked by peptide inhibitors such as ICE/CED-3-like caspases (3), indicating that c-Jun induces apoptosis indirectly through protease intermediates.

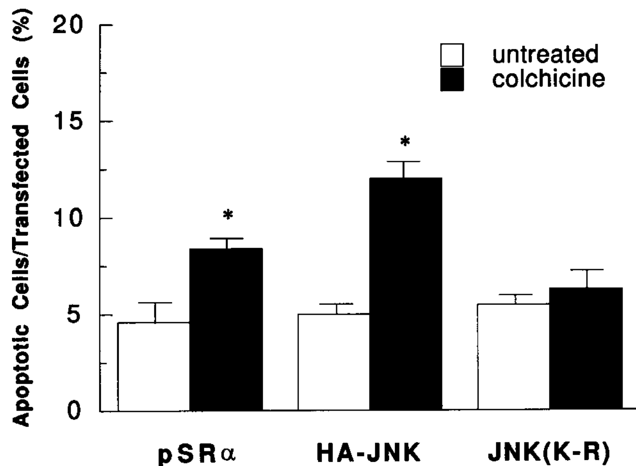

Fig. 7. Effects of 10^{-6} M colchicine on percentage of apoptotic cells among transfected cells for cells cotransfected with pSRα, HA-JNK, and JNK(K-R), as studied by using triple-staining technique exemplified in Fig. 6. For each group, results were obtained from ≥3 independent experiments, with 150 transfected cells counted in each experiment. Values are means ± SD.

Our finding that laminar shear stress does not induce apoptosis is in agreement with the concept that laminar shear stress is protective for EC integrity. This concept is promulgated by Dimmeler et al. (10) on the basis of their finding that shear stress protects ECs from being driven into apoptosis on exogenous stimulation with tumor necrosis factor-α or withdrawal of survival factors. One of the long-term responses of ECs to laminar shear stress is cytoskeletal reorganization (12). From this point of view, the enhanced MT organization by shear stress is protective against apoptosis, whereas the MT disruption by colchicine leads to apoptosis. Thus the integrity and function of the MTs may play an important role in the homeostatic regulation of the cell cycle.

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