Altered reactivity of hemolysate-treated cultured smooth-muscle cells from rabbit basilar artery determined by digital imaging microscopy

YOSHIHIRO TAKANASHI, M.D., KAZUHIKO FUJITSU, M.D., SATOSHI FUJI, M.D., AND TAKEO KUWABARA, M.D.

Department of Neurosurgery, Yokohama City University School of Medicine, Yokohama, Japan

During culture, smooth-muscle cells obtained from rabbit basilar arteries were examined for contractile activity by means of differential interference microscopy with a video analysis system (digital imaging microscopy system). This system proved useful for observing the contraction and ultrastructural changes of the living cells. Hemolysate-treated cells showed augmented responses to 5-hydroxytryptamine and leukotriene C4, but not to KCl. This augmented response diminished gradually during the culture period. Both a phospholipase C blocking agent, 2-nitro-4-carboxyphenol-n,n-diphenylcarbamate (NCDC), and a myosin light chain kinase blocking agent, 1-(5-chloronaphthalenesulfonyl)-1H-hexahydro-1,4-diazepine (ML-9), suppressed this augmented response. Protein kinase C activity of the cells, as measured by Western blot analysis, did not increase during the period of culture with hemolysate. The results obtained suggest that hemolysate exerts the following effects on the cells: 1) acute but gradual contraction of the cells; 2) augmentation of cellular responses to vasoactive agents; and 3) progressive contraction and morphological alteration of the cells. Possible mechanisms by which hemolysate exerts these effects are discussed, taking into consideration the interrelationship between these effects.

KEY WORDS • cell culture • vasospasm • hemolysate • protein kinase C • myosin light chain kinase • smooth-muscle cell • rabbit

A model for chronic in vitro experimentation on cerebral vasospasm using cultured arterial smooth-muscle cells from the rat aortic media was described in 1988. Smooth-muscle cells showed progressive contraction and ultrastructural changes when cultured in a medium with hemolysate. Because these results were reached with electron microscopic observation, the question as to whether the smooth-muscle cells retained contractile reactivity throughout the period of culture was not resolved. To observe chronic as well as acute contraction of the living cells, a differential interference microscope with a video analysis system (digital imaging microscopy system) was used in this study. Another improvement upon the experimental model was that the smooth-muscle cells were cultured from rabbit basilar arteries. Many workers have reported that subarachnoid hemorrhage (SAH) alters the reactivity of the cerebral artery. With the system and model described, the reactivity of hemolysate-treated smooth-muscle cells has been investigated during an 8-day period. Several agents that modulate the cell contractile process and measurements of protein kinase C activity were used to explore the possible mechanisms by which hemolysate exerts its effects on arterial smooth-muscle cells in culture.

Materials and Methods

Culture of Smooth-Muscle Cells

Smooth-muscle cells were cultured by an enzyme-dispersal method from the basilar artery of male Japanese White rabbits, each weighing 2.0 to 2.5 kg. The basilar artery was cut into small pieces, then treated by the following enzymatic disaggregation. First, the fragments were placed into a centrifuge tube containing 0.2% collagenase and 0.2% trypsin in Hanks' balanced salt solution and were incubated for 45 minutes at 37°C in an atmosphere of 95% air and 5% CO2. The culture medium used was Dulbecco's modified Eagle's medium, Ham F-10, and 10% fetal calf serum, containing 100 μL/ml of penicillin and 100 μg/ml of streptomycin. In approximately 2 weeks, the cells were nearly conflu-
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Fig. 1. Upper: Experimental design. A coverglass holding smooth-muscle cells was fixed with thin waterproof tape to the bottom of a stainless steel plate (50 × 80 × 0.6 mm) with an elliptical 30 × 35-mm hole. The top of the stainless steel plate was covered with a coverslip, creating a small chamber for the cells and leaving small openings (inlet and outlet) on both sides for the solution to pass through. Lower: Digital imaging microscopic system. The cells were imaged using a Zeiss Plan 40×/0.65 numerical aperture objective and a Zeiss IM-35 inverted microscope equipped with Nomarski differential interference optics. The image of the cells was captured with a Chalcogen camera driven by an Argus 100 image processor and was visualized on a recording display. The cell areas were calculated by a C-2117 video manipulator.

ent, after which they were subcultured for another 2 weeks until they reached confluence. The cells from the second culture were seeded at 5 × 10^5 cells/ml on coverslips. These coverslips were placed on plastic dishes which were then put in a tissue incubator. The viable cells on the coverslips were observed by phase-contrast microscopy and Nomarski optics. For immunofluorescence staining, the cells were stained with a fluorescein isothiocyanate (FITC)-labeled antibody against smooth-muscle actin and myosin.

Experimental Design for Digital Imaging Microscopy System

A coverslip with the cultured cells was fixed with thin waterproof tape to the bottom of a stainless steel plate 0.6 mm thick with an elliptical hole. The top of the stainless steel plate was covered with a coverslip, creating a small chamber for the cells and leaving small openings on both sides for the addition of experimental solutions (Fig. 1). This plate was placed on a Zeiss IM-35 inverted microscope equipped with Nomarski differential interference optics. The contractile response of the smooth-muscle cells was tested by adding vasoactive agents directly to this small chamber. The digital imaging microscopy system was developed to observe living cells and to record their contractile response on videotape. The cell area was measured by a video manipulator, and contraction was expressed as the percentage reduction in cell area (Fig. 2). The cell area was measured both before and 5 minutes after administration of vasoactive agents, and hemolysate-induced contraction was measured 15 minutes after administration. The contractile percentage in this study was assessed by means of the following formula: 100 × (control area - experimental area)/control area.

Values are expressed as the mean ± standard error of the mean. Statistical significance was determined using Student's t-test, and probabilities of less than 5% (p < 0.05) were considered significant.

Hemolysate and Vasoactive Agents

The hemolysate was prepared as follows. The red blood cells from a rabbit were washed three times in 0.9% saline and allowed to lyse at -70°C. At 4°C, and under physiological osmotic pressure, the ghost-free supernatant of the hemolysate was dialyzed against normal saline. The concentration of the hemolysate was expressed as the hemoglobin content, which was...
determined by spectrophotometric absorption spectra. The other agents used in this study were as follows: 5-hydroxytryptamine (5-HT); leukotriene C₄ (LTC₄); the phospholipase C blocking agent¹⁸ 2-nitro-4-carboxyphenyl-n,n-diphenylcarbamate (NCDC); the myosin light chain kinase blocking agent¹⁵¹⁶ 1-(5-chloronaphthalenesulfonyl)-1H-hexahydro-1,4-diazepine (ML-9); and the protein kinase C activating agent²³²⁷³⁴ 12-O-tetradecanoyl-phorbol-13-acetate (TPA).

Smooth-Muscle Cell Contraction

Preliminary observations were made to see if the digital imaging microscopy system was capable of measuring contraction and if the cells showed dose-dependent contractile responses to the following vasoactive agents: LTC₄ (10⁻⁸ to 10⁻¹⁵ M), 5-HT (10⁻⁵ to 10⁻¹¹ M), and hemolysate (10⁻⁴ to 10⁻⁹ M). The time course of the cell contraction caused by hemolysate was also examined. For this purpose, the cell area was compared between hemolysate-treated cells and the control cells (cultured in medium only) for an 8-day period.

Effect of Hemolysate on Smooth-Muscle Cell Reactivity

Cultured smooth-muscle cells were divided into two groups. The first group was cultured in medium only (control group), and the second group was cultured in the medium containing 10⁻⁴ M hemolysate (hemolysate-treated group). Six hours after the start of the experiment on Day 0, the medium was replaced with fresh medium of the same composition with or without the same concentration of the hemolysate. In each group, the contractile response to 10 ng/ml of LTC₄, 10⁻⁵ M of 5-HT, or 50 mM of KCl was followed during an 8-day culture period.

Effect of NCDC and ML-9 on Hemolysate-Treated Cell Reactivity

First, ML-9 (14 μM) or NCDC (10 μM) was examined in acute experiments on cells contracted by 10⁻⁴ M of hemolysate, 10⁻⁵ M of 5-HT, or 10 ng/ml of LTC₄. Second, cultured smooth-muscle cells were divided into three groups. Each group was cultured in a medium containing 10⁻⁴ M hemolysate; no agent was added to the first group, 10 μM of NCDC was added to the second group, and 14 μM of ML-9 was added to the third group. At 6 hours, the medium was replaced with fresh medium of the original composition. In each group, the contractile responses to LTC₄, 10 ng/ml, or 10⁻⁵ M of 5-HT were tested over a 3-day period of culture.

Measurement of Protein Kinase C Activity

To measure protein kinase C activity, cultured smooth-muscle cells were divided into three groups. The cells in the first group were cultured in the normal medium only (control group). The cells in the second group were cultured in the medium containing 1 nM, 100 nM, or 1000 nM TPA for 1 hour (TPA-treated group). The cells in the third group were cultured in a medium containing 10⁻⁴ M hemolysate (hemolysate-treated group) and were followed for 6 days. The medium was changed in the same way as in the previous experiments. On Days 0, 2, 4, and 6, the cells in the control group, the TPA-treated group, and the hemolysate-treated group were gently detached and collected. They were homogenized in 20 mM Tris (pH 7.5), 2 mM ethylenediaminetetra-acetic acid (EDTA), 0.25 M
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Fig. 3. Immunofluorescent staining of smooth-muscle cells. Bars = 10 μm. *Left Pair: Actin studies of smooth-muscle cells after 4 days in the second culture: Nomarski imaging (upper) and FITC-labeled antibody staining against smooth-muscle actin (lower). Right Pair: Myosin studies of smooth-muscle cells after 4 days in the second culture: Nomarski imaging (upper) and FITC-labeled antibody staining against smooth-muscle myosin (lower).*

sucrose, 0.5 mM ethyleneglycol-bis-(β-aminoethylether)-N,N'-tetra-acetic acid (EGTA), 50 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 100 μg/ml of leupeptin. To obtain the cytosol fraction protein kinase C, the homogenates were spun at 100,000 G for 30 minutes and the supernatants were collected. To obtain the membrane fraction protein kinase C, the corresponding precipitates were resuspended in 20 mM Tris (pH 7.5), 2 mM EDTA, 0.25 M sucrose, 0.5 mM EGTA, 50 mM 2-mercaptoethanol, 2 mM PMSF, 100 μg/ml of leupeptin, and 0.1% Triton X-100. The solutions were centrifuged at 100,000 G for 30 minutes to remove debris, and the supernatants were collected. The supernatants from these two preparations were applied separately to an 8.5% polyacrylamide slab gel 1.0 mm thick for sodium dodecyl sulfate-polyacrylamide gel electrophoresis.14,32 Proteins on the gel were electrophoretically transferred to nitrocellulose paper at 11.4 V/cm for 3 hours.14,32 After transfer, the nitrocellulose paper was rinsed with Tris-NaCl buffer (50 mM Tris/HCl pH 7.6) and 135 mM NaCl then incubated for 1 hour at 37°C with Tris-NaCl buffer containing 3% (wt/vol) bovine serum albumin (BSA) to block any remaining binding sites. The nitrocellulose paper was incubated overnight at 4°C with monoclonal anti-protein kinase C* in Tris-NaCl buffer containing 3% BSA. After being washed with Tris-NaCl buffer, the nitrocellulose paper was incubated for 60 minutes with biotinylated goat anti-mouse immunoglobulins with normal serum. The nitrocellulose paper was washed as before and incubated for 60 minutes with alkaline phosphatase-conjugated streptavidin. After another wash with Tris-NaCl buffer, the paper was incubated with a solution containing 1 mg/ml of naphthol AS-BI phosphate and 1 mg/ml of Fast Red TR salt in Tris/HCl (pH 8.2). Development of the colored band was stopped by rinsing the paper in distilled water.

Results

Smooth-Muscle Cells in Culture

Phase-contrast microscopy observation of the cells demonstrated the "hills and valleys" appearance which is the characteristic growth pattern in a confluent culture of smooth-muscle cells. Under high-power magnification with the Nomarski differential interference microscope, smooth-muscle cells had a large number of myofilaments in the cytoplasm. All of the cells from the rabbit basilar artery that were characterized as smooth-muscle cells by both phase-contrast microscopy and Nomarski optics reacted intensely with the anti-smooth-muscle actin and myosin (Fig. 3). Fibroblasts from the rabbit aortic adventitia, on the other hand, were very weakly stained with these antibodies.

Dose-Dependent Contractile Response of Cultured Smooth-Muscle Cells

The contractile responses of smooth-muscle cells to different doses of 5-HT, LTC₄, or hemolysate are shown.
in Fig. 4. Each vasoactive agent caused a dose-dependent contractile response of the cells, with LTC₄ producing the most prominent contraction. The contraction caused by hemolysate was more gradual and more long-lasting than that caused by other agents.

Fig. 4. Dose-response curves of cell contracture caused by 5-hydroxytryptamine (5HT), leukotriene C₄ (LTC₄), or hemolysate application. Vertical bars represent mean ± standard error of the mean. Numbers in parentheses indicate the number of preparations tested.

Fig. 5. Cell area correlated with culture time before applying each vasoactive agent to the control group and the hemolysate-treated group. Vertical bars represent mean ± standard error of the mean. Numbers in parentheses indicate the number of preparations tested. ** = p < 0.01 different from the control group by Student's t-test.

Fig. 6. Augmenting effect of hemolysate pretreatment on contractile response to vasoactive agents during an 8-day period. Contractile responses to 10 ng/ml leukotriene C₄ (LTC₄) (a), 10⁻⁴ M 5-hydroxytryptamine (5HT) (b), and 50 mM KCl (c). For hemolysate-pretreated cells, 10⁻³ M hemolysate was added to the medium on Day 0 of the experiment. The contractile response to LTC₄ and 5HT was augmented by hemolysate pretreatment while the contractile response to KCl was not. Vertical bars represent mean ± standard error of the mean. Numbers in parentheses indicate the number of preparations tested. Difference from control group: * = p < 0.05 and ** = p < 0.01 by Student's t-test.
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**Time Course of Hemolysate Cell-Induced Contraction**

Figure 5 shows the time course of cell contraction caused by hemolysate. During the culture period, some smooth-muscle cells detached from the coverslips, probably because of cell death. These floating cells were discarded when the medium was changed, and the following observations were conducted only with the cells on the coverslips. In the first 3 days of culture, there was no significant difference between the average cell area of the hemolysate-treated group and that of the control group; however, the average cell area was significantly decreased in the hemolysate-treated group on Days 5 and 8 of culture.

![Graph showing time course of hemolysate cell-induced contraction](image)

**Effect of Hemolysate Treatment on Smooth-Muscle Cell Reactivity**

As shown in Fig. 6a and b, both LTC₄ and 5-HT produced more prominent contraction in the hemolysate-pretreated cells than in the cells of the control group. This augmenting effect progressively diminished with time in culture. Hemolysate pretreatment did not show any augmenting effect on cell contraction produced by KCl (Fig. 6c).

**Suppression by ML-9 and NCDC of Augmented Cell Contraction**

In the acute experiment, the contractile response to each vasoactive agent was suppressed by both ML-9 and NCDC (Fig. 7a). In the chronic experiment (3 days), the augmenting effect of hemolysate pretreatment on cell contraction produced by LTC₄ was substantially suppressed by both ML-9 and NCDC (Fig. 7b). The augmented contraction in response to 5-HT was also suppressed by ML-9 and NCDC (Fig. 7c).

**Protein Kinase C Activity in the Hemolysate-Treated Cells**

Figure 8 shows the results of the Western blot analysis. There was no change in protein kinase C activity of either the cytosol or membrane fraction following stimulation with 1 nM TPA for 1 hour (data not shown), whereas treatment with 100 nM TPA for 1 hour resulted in a prominent decrease in the cytosol fraction (Fig. 8 arrow). However, no definite increase in the immunoreactive band in the membrane fraction was observed.

![Graph showing protein kinase C activity](image)

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Fig. 7. a: The inhibitory effect of 10 μM NCDC and 14 μM ML-9 on contraction induced by 10⁻⁴M hemolysate, 10⁻⁴M 5-hydroxytryptamine (5HT), and 10 ng/ml leukotriene C₄ (LTC₄) in an acute experiment. b and c: Suppressing effect of 10 μM NCDC and 14 μM ML-9 on the augmentation by hemolysate over a 3-day period of observation. Contraction was obtained in response to 10 ng/ml of LTC₄ (b) and 10⁻⁴ M of 5HT (c). Vertical bars represent mean ± standard error of the mean. Numbers in columns and parentheses indicate the number of preparations tested. ** = p < 0.01.
in 100 nM TPA-treated cells, and 1000 nM of TPA caused detachment from the culture dishes in approximately 50% of the cells. No significant change in protein kinase C activity was observed in the hemolysate-treated cells on any day of culture (Fig. 8).

**Discussion**

**Culture of Vascular Smooth-Muscle Cells**

The response of cells to any vasoactive factor is extremely difficult to assess in vivo; therefore, culture of smooth-muscle cells is a useful method for studying their involvement in cerebral vasospasm. However, smooth muscle in cell culture has special features and technical problems that must be considered.

Smooth-muscle cells change their phenotype in culture in response to altered functional demands. To accomplish this multiplicity of functions, smooth-muscle cells can exist in a whole spectrum of phenotypes. One phenotype is the smooth-muscle cell with the almost exclusive function of contraction. Another phenotype is the smooth-muscle cells whose function is almost exclusively that of synthesis. Fluoresceinated antibodies against smooth-muscle myosin will stain contractile smooth-muscle cells intensely but have little or no reaction with either synthetic smooth-muscle cells or fibroblasts. However, with fluoresceinated antibodies against smooth-muscle actin, synthetic smooth-muscle cells have the same intense staining reaction in their fibrils.

Two methods of culture are employed in the majority of reports. One method of culture is to place small explants of arterial media into a culture chamber until the cells become confluent. In a previous study, this method was used for the rat aortic media. Smooth-muscle cells obtained by the explant method have little response to vasoconstrictor agents. At confluence, a small amount of myosin immunofluorescence is gained but the cells cannot be induced to contract. Therefore, in the previous study, smooth-muscle cells were seeded so densely (10⁶ cells/ml) that they could remain in the contractile state because there was a change in smooth-muscle phenotype that was dependent on initial cell seeding density.

Another method of culture, as described in this paper, is dissociation of smooth muscle by the action of enzymes. An enzyme-dispersal method is the optimal culture procedure to obtain smooth-muscle cells of contractile properties. As described in this report, these cells contain thick myofilaments and they can be induced to contract with vasoconstrictor agents. They stain intensely with FITC-labeled antibodies against smooth-muscle actin and myosin. Low-magnification studies with the digital imaging microscopy system showed no obvious morphological difference between the rat aortic smooth-muscle cells and the rabbit basilar artery smooth-muscle cells in culture. Likewise, there was no obvious difference in the morphological characteristics between the contraction caused by hemolysate and that caused by other vasoconstrictor agents. Under higher magnification, the cytoskeletal architecture and intracellular particles were observed using the digital imaging microscopy system. Such studies are under way but are beyond the scope of the present work. The digital imaging microscopy system is considered useful for future investigation of the relationship between contraction and ultrastructural changes of smooth-muscle cells.

**Altered Reactivity of Hemolysate-Treated Cells**

Whether the contractile reactivity of isolated cerebral arteries is increased or decreased after SAH is still controversial. Some workers have reported that 5-HT produced a decreased response in cerebral arteries after SAH, whereas others have suggested an increased response. The response to potassium was reported to decrease in monkeys and dogs but increase in rabbits. There have been few reports on the cerebral arterial response to LTC₄ after SAH. The present observations proved that the hemolysate-pre-treated cells showed increased responses to 5-HT and LTC₄. These increased responses were not considered to be simply additive with hemolysate because no combination of other vasoactive agents showed increased contraction (not shown). Hemolysate, therefore, was considered to augment the cells' contractile responses to vasoactive agents. This augmenting effect diminished progressively with time in culture. Progressive diminution in the augmenting effect may be attributable to progression of cell contraction caused by hemolysate. The potential for contraction, therefore, appears to diminish progressively as the cells contract.

**Mechanism of Altered Reactivity of Cells**

Some workers have reported that blockade of the voltage-dependent Ca²⁺ channel causes hypersensitivity to the receptor agonists. The augmenting effect of hemolysate pretreatment, however, is not interpreted to indicate the blockade of the voltage-dependent Ca²⁺ channel because hemolysate pretreatment did not show any effect on the contraction caused by KCl. Therefore, it may be that the intracellular signal transduction invoked by potassium is different from that induced by receptor agonists. Receptor-mediated activation of phospholipase C triggers the cascade of inositol phospholipid breakdown, causing intracellular Ca²⁺ mobilization. The Ca²⁺-calmodulin complex activates myosin light chain kinase, resulting in myosin phosphorylation and contraction of smooth-muscle cells. Both the phospholipase C blocking agent NCDC and the myosin light chain kinase blocking agent ML-9 suppressed the contraction caused by hemolysate and the augmenting effect of hemolysate. These results suggest that the acute contraction induced by hemolysate is caused either by affecting the receptor-operated Ca²⁺ channel or by promoting the inositol phospholipid breakdown. They also suggest that the suppressing effect of NCDC and ML-9 on the augmenting effect
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![Diagram of protein kinase C activation with Cytosol and Membrane fractions]

**Fig. 8.** Western blot analysis of protein kinase C with a monoclonal antibody. Protein kinase C activity in the membrane and cytosol fraction did not differ significantly between the control group and the hemolysate-treated group. Treatment with 100 nM 12-O-tetradecanoyl-phorbol-13 acetate (TPA) for 60 minutes resulted in a prominent decrease of the cytosol fraction (arrow).

of hemolysate is secondary to blocking the hemolysate-induced contraction. The exact mechanism of the augmenting effect of hemolysate treatment remains obscure, but this augmenting effect, combined with multiple vasoactive agents produced by SAH, may lead to a vicious circle of progressive vasospasm.

**Effects of Hemolysate on Cultured Arterial Smooth-Muscle Cells**

The hemolysate prepared for this study is rich in oxyhemoglobin; however, other factors may also exist in this hemolysate. The previously described effects of hemolysate, therefore, may be the results of an interaction between oxyhemoglobin and other factors not yet defined. Judging from the results obtained in both the previous and present studies, hemolysate appears to have three effects on cultured arterial smooth-muscle cells: 1) acute but gradual contraction of the cells; 2) augmentation of cellular response to vasoactive agents; and 3) progressive contraction and ultrastructural alteration of the cells. Serial observation with the digital imaging microscopy system proved that the cells on the coverslips retained contractile reactivity in spite of their progressive contraction. However, some of the cells detached from the coverslips, probably because of cell death.

The mechanism by which hemolysate exerted the third effect is least well understood. The possibility was explored that hemolysate affects protein kinase C activity in the presence of diacylglycerol, which is produced by receptor-mediated hydrolysis of inositol phospholipids. In Western blot analysis, a decrease in the cytosol fraction combined with an increase in the membrane fraction (translocation) is considered to indicate the increased activity of protein kinase C. The protein kinase C activating agent TPA is believed to cause this translocation. In our experiment, however, 100 nM TPA only caused a decrease in the cytosol fraction without any increase in the membrane fraction. This is probably due to either the small number of smooth-muscle cells or the low concentration of TPA. In the present culture system, larger numbers of cells cannot be obtained and a higher concentration of TPA is toxic to the cells, causing detachment from the culture dishes. However, the decrease in the cytosol fraction was considered crucial, even if there was no corresponding increase in the membrane fraction. Hemolysate-treated cells showed no significant change in the immunoreactive band of protein kinase C on any day of culture. The exact role of protein kinase C is still unclear and some workers have reported that the principal role of protein kinase C is to regulate or control the contraction of smooth-muscle cells. The possibility cannot be excluded, therefore, that hemolysate disturbs these regulatory roles of protein kinase C, leading to the pathological contraction and myonecrosis of the cells.

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