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Hemolysate Induces Tyrosine Phosphorylation and Collagen-Lattice Compaction in Cultured Fibroblasts

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Hemolysate, a proposed causative agent for cerebral vasospasm after subarachnoid hemorrhage, produces contraction of cerebral arteries by activation of tyrosine kinases. In addition, hemolysate increases fibroblast-collagen compaction that could play a role in cerebral vasospasm. We studied the effect of hemolysate on tyrosine phosphorylation and fibroblast-collagen compaction in cultured canine basilar and human dermal fibroblasts using tyrosine kinase inhibitors and tyrosine antibodies. Hemolysate enhanced tyrosine phosphorylation of two proteins, 64 and 120 kDa, in cultured canine basilar artery and human dermal fibroblast cells. The effect of hemolysate was time-dependent and concentration-dependent. Oxyhemoglobin and ATP, the two major components of hemolysate, produced similar tyrosine phosphorylation, however, with a different time course. Tyrosine kinase inhibitors genistein and tyrphostin A51 abolished the effect of hemolysate in both cerebral and dermal fibroblasts. Hemolysate increased fibroblast-populated collagen-lattice compaction and tyrosine kinase inhibitors genistein and tyrphostin A51 attenuated the effect of hemolysate. We conclude that hemolysate activates tyrosine kinase that leads to the increase of fibroblast compaction. This effect of hemolysate may contribute to cerebral vasospasm. © 1999 Academic Press

Key Words: **tyrosine phosphorylation; hemolysate; collagen-lattice compaction; cerebral vasospasm.**

Cerebral vasospasm, a persisted narrowing of major cerebral arteries, is a major cause of morbidity and mortality following subarachnoid hemorrhage (SAH) (11, 13, 15). The etiological factors for cerebral vasospasm are subarachnoid blood clots, especially the lysate of erythrocyte (2, 17, 18). However, the pathogenesis of cerebral vasospasm and the signal transduction pathways responding to the spasmogens remain unclear (7, 15). Recently, tyrosine kinases and their substrates have been suggested involved in hemolysate-induced elevation of intracellular Ca^{2+} (9), in contraction of cerebral arteries (12, 24, 30), and in a canine model of cerebral vasospasm (4).

Even though cerebral vasospasm is regarded as a prolonged contraction of major cerebral arteries, there is a line of evidence suggesting additional or alternative mechanisms (3, 21). First, cerebral vasospasm has been resistant to all known vasodilators. Second, the most striking histological feature of the spastic vessels is the thickening of the sub-endothelial layer called subintimal cellular proliferation. Third, the cellular nature of those proliferative areas has the properties similar to myofibroblasts. Furthermore, cerebrospinal fluid obtained from patients with ruptured aneurysm significantly accelerated collagen-lattice contraction, especially when the patient developed symptomatic vasospasm (21, 25). This suggests that non-muscle components can produce and maintain vascular constriction (10, 19, 26).

Thus, we studied the effect of hemolysate and its components on tyrosine phosphorylation and fibroblast-collagen compaction in cultured canine basilar artery and human dermal fibroblasts.

MATERIALS AND METHODS

Cell culture. Neonatal normal human dermal fibroblast cells (NHDF-Neo) were purchased from Clonetics (San Diego, CA). Cells were cultured in FBM medium, supplemented with 2% fetal bovine serum, 1 ng/ml basic human fibroblast growth factor, antibiotics (gentamycin 50 μg/ml, amphotericin-B 50 ng/ml) and 5 μg/ml insulin in a 5% CO_2 incubator.

Canine basilar arterial fibroblast cells were obtained using explant methods (9) and cultured in Dulbecco's modified Eagle's medium (Gibco Brl, Grand Island, NY) with 10% fetal bovine serum. These cells were stained negative to factor VIII and α-smooth muscle actin. Cells from the 3rd and 8th passages were used.

Preparation of hemolysate. Hemolysate was prepared from fresh arterial dog blood in our laboratory as described (9). Briefly, dog red blood cells were washed with cold saline solution and lysed by adding 5 mM cold sodium phosphate buffer. The membrane debris was

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pelleted by centrifugation at 31,000*g* for 15 min and the erythrocyte lysate was stored at −80°C. The concentrations of oxy- and methemoglobin were determined spectrophotometrically. The hemoglobin (100% oxyhemoglobin) concentration in hemolysate was 10.5 mM. The concentration of adenosine triphosphate (ATP) was 30 μM, measured by HPLC.

Phosphotyrosine immunoblot analysis. The method for Western blot was described previously (9). Cells were grown to confluency in 60 mm culture dishes and changed to serum free FGM for 24 h before experiments. Hemolysate was added to the dishes for different designated times at 37°C in humidified 95% O_2 and 5% CO_2. The medium was quickly removed and the dishes were washed twice with 4 ml ice-cold Hanks' balanced salt solution, containing 1 mmol/L sodium orthovanadate. The cells were collected with 300 μl lysis buffer (0.1 mol/L NaCl, 0.02 mol/L Tris, 10% glycerol, 1% NP-40, pH 8.0) with 1 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulphonyl fluoride and 10 μg/ml aprotinin (Sigma Chemical Co., St. Louis, MO). Cell lysates were centrifuged at 13,2000*g* for 10 min at 4°C to remove insoluble debris and solubilized for SDS-PAGE. Protein concentration was determined by using BSA as a standard. Protein samples were mixed with an equal volume of SDS-PAGE sample buffer (5×) and were boiled for 3 min. Equal protein concentrations (30 μg) of the lysate were subjected to 10% (wt/vol) SDS-PAGE, transferred to nitrocellulose membranes (Transblot, Bio-Rad Laboratories, CA), and blotted with an antiphosphotyrosine monoclonal antibody (dilution 1:1000, 4G10, Upstate Biotechnology Incorporated Lake Placid, NY). Phosphorylated proteins were visualized using a horseradish peroxidase-conjugated goat antibody against mouse IgG (dilution 1:1000, Southern Biotechnology Associates, Inc., USA) and enhanced by chemiluminescence reagents (ECL RPN2209, Amersham Pharmacia Biotech, England, UK). The results were quantified by laser densitometry of the films and integrated whole band analysis (Molecular Dynamics, Image Quant, Sunnyvale, CA).

Fibroblast-populated collagen lattices (FPCL) compaction. The method for FPCL compaction was described (19). Fibroblast-populated collagen lattices (FPCLs) were formed by mixing trypsin-liberated fibroblasts in DMEM (1.6 ml) with a sterile stock solution of acetic acid-extracted rat tail tendon collagen (Type I) (0.4 ml of 5 mg/ml in 1 mmol/L HCl) in 12 × 75-mm polystrene tubes with a vortex mixer. The mixture was immediately transferred to 16 mm wells (24-well plates) and allowed to gel at 37°C. The repolymerization process typically occurred within minutes, trapping the cells in the resulting lattice matrix. Liberated cells were counted using a hemocytometer and were diluted with DMEM to provide 70,000 cells/ml in each lattice. To ensure even contraction, each FPCL was freed from the dish walls and surface with a fine needle after formation.

Measurement of lattice compaction. To test the effect of hemolysate and tyrosine kinase inhibitor on lattice compaction, 0.1 ml of hemolysate and tyrosine kinase inhibitors was added after the lattice was freed. During incubation, the fibroblasts progressively compacted by the collagen fibrils in all three dimensions in a process known as lattice contraction. At 24-h intervals after the addition of hemolysate, lattice contraction was determined as a reduction in area. Lengths of the longer and shorter axes of each contracting lattice were measured using a scale of graph paper beneath the dish. The areas were then calculated, considering each lattice as an ellipse. Each treatment was done in triplicate.

Data analysis. Data are expressed as means ± SE. Statistical differences between the control and other groups were compared by analysis of variance (ANOVA), and a value of $P < 0.05$ was considered statistically significant.

RESULTS

Tyrosine Phosphorylation to Hemolysate

Hemolysate at a concentration of 10% by volume was added to fibroblast cells in serum free media for times up to 60 min. The cells were lysed and the levels of tyrosine phosphorylation of cellular proteins at different time points were determined using Western blotting (Fig. 1A). Hemolysate was found to enhance tyrosine phosphorylation of two proteins of approximately 64 and 120 kDa. Both cultures of fibroblasts, canine basilar artery (Fig. 1A) and human dermal (Fig. 1B), showed reproducible changes in tyrosine phosphorylation. The effect of hemolysate was time-dependent, the maximum effect was observed 5 min after the treatment, and the phosphorylation lasted for more than 60 min. Figure 1C summarizes results from 6 experiments in human dermal cells.

Cells were exposed for 5 min to serial dilutions (0.1, 1, 10, 50%) of hemolysate to establish a concentration-dependent response of tyrosine phosphorylation. Figure 2 shows that there was increased tyrosine phosphorylation with each increase in concentration of hemolysate, and the increase in staining of the two bands was nearly identical at all concentrations. At the highest hemolysate concentration (50%), the level of tyrosine phosphorylation was approximately 2.7 times that of the untreated cells (control).

Tyrosine Phosphorylation to Oxyhemoglobin and ATP

Two major components of hemolysate, oxyhemoglobin and ATP, were studied separately. Oxyhemoglobin (10 μmol/L) was added to human dermal fibroblast cells in serum free media for times up to 60 min. Oxyhemoglobin was found to enhance tyrosine phosphorylation of two proteins of approximately 64 and 120 kDa, similar to that of hemolysate (Fig. 3A). The effect of oxyhemoglobin was time-dependent in that the peak response was obtained at 3 min and a second peak was obtained at 60 min. Under the same condition, ATP (10 μmol/L) enhanced tyrosine phosphorylation of the same two bands in a similar time-dependent fashion in human dermal fibroblasts (Fig. 3B). The peak response was obtained at 3 min and a second peak was obtained at 60 min. The experiments of oxyhemoglobin and ATP on tyrosine phosphorylation were repeated once each in canine basilar artery fibroblasts and the results were similar to those in human dermal cells (not shown).

Tyrosine Kinase Inhibitors

Pre-incubation with tyrosine kinase inhibitors, genistein (30 μmol/L) and tyrphostin A51 (30 μmol/L), for 60 min, markedly ($P < 0.05$, Fig. 4A–B) reduced the

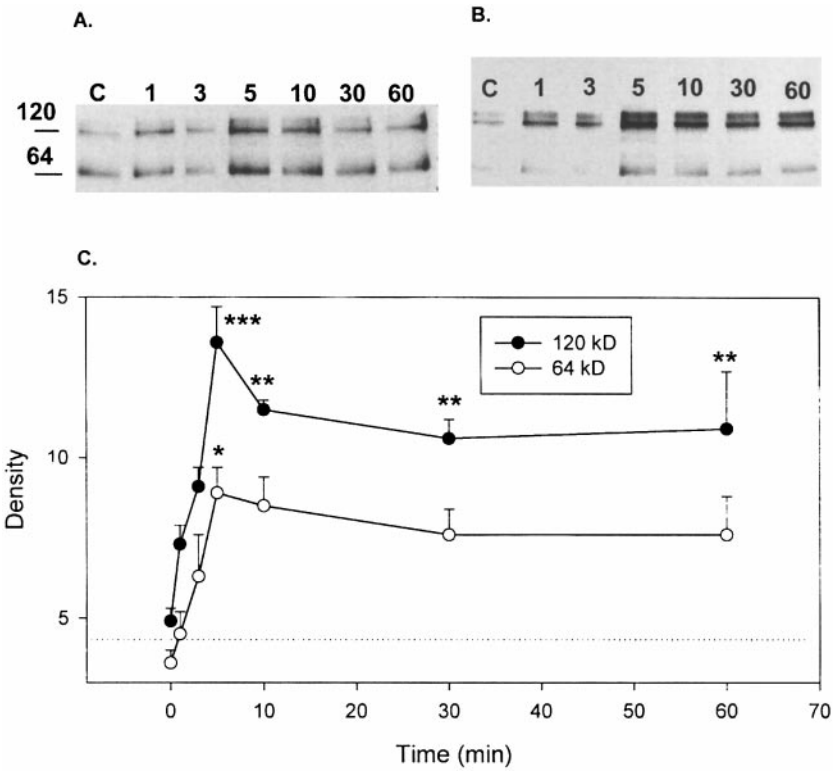

FIG. 1. Time course of hemolysate-induced tyrosine phosphorylation in cultured canine basilar arterial and human dermal fibroblasts. Western blot stained with monoclonal antibody to phosphotyrosine. (A) Hemolysate (10%) was added to canine basilar arterial fibroblast cells for the indicated times. Equal amounts of protein were loaded into each lane. Hemolysate enhanced tyrosine phosphorylation of the 120 and 64 kDa proteins in a time-dependent fashion. The peak response to hemolysate was observed at 5 min and then the signal decayed slightly and maintained an elevated level above the resting level up to 60 min. (B) A similar study was repeated in human dermal fibroblasts. Hemolysate enhanced the tyrosine phosphorylation of the 120 and 64 kDa proteins, the same bands as in canine basilar arterial fibroblasts, in a time-dependent manner. The response started at 1 min, peaked at 5 min, and lasted up to 60 min. (C) Summary of 6 experiments using human dermal cells. Data were quantified by a densitometer and the laser density was calculated and plotted. *, **, and *** indicate $P < 0.05$, 0.011, and 0.001, respectively (ANOVA). "C" indicates control, 1, 3, 5, 10, 30, and 60 are the incubating times (min) with hemolysate (10%).

effect of hemolysate (10%, 5 min treatment) on tyrosine phosphorylation in cultured canine basilar artery fibroblast cells. Figure 4B summarizes the effect of tyrosine kinase inhibitors, either in the presence or in the absence of hemolysate. In human dermal fibroblasts, hemolysate significantly increased tyrosine phosphorylation and pre-incubation with genistein and tyrphostin A51 (30 μmol/L) abolished the effect of hemolysate under similar conditions as in cerebral cells (not shown).

Fibroblast-Populated Collagen-Lattice (FPCL) Compaction

In another series studies, addition of 10% hemolysate into the three-dimensional collagen matrix increased significantly fibroblast (dermal) collagen compaction compared to untreated cells. Hemolysate accelerated FPCL compaction on days 1, 2, and 3 with statistical significance compared to the corresponding days with the control. Pre-incubation of tissues with tyrosine kinase inhibitors genistein (30 μmol/L) (Fig. 5) and tyrphostin A51 (30 μmol/L) reduced the effect of hemolysate. All experiments were repeated five times with 10 duplicates in each study.

DISCUSSION

There are four principal findings in this study. (1) Hemolysate enhanced tyrosine phosphorylation in cultured canine basilar artery and human dermal fibroblast cells. The effect of hemolysate was time-dependent and concentration-dependent. (2) Oxyhemoglobin and ATP produced similar tyrosine phosphorylation, however, with a different time course. (3) Tyrosine kinase inhibitors genistein and tyrphostin A51 abolished the effect of hemolysate in both cerebral and dermal fibroblasts. (4) Hemolysate increased fibroblast populated collagen-lattice compaction and tyrosine kinase inhibitors genistein and tyrphostin A51 attenuated the effect of hemolysate.

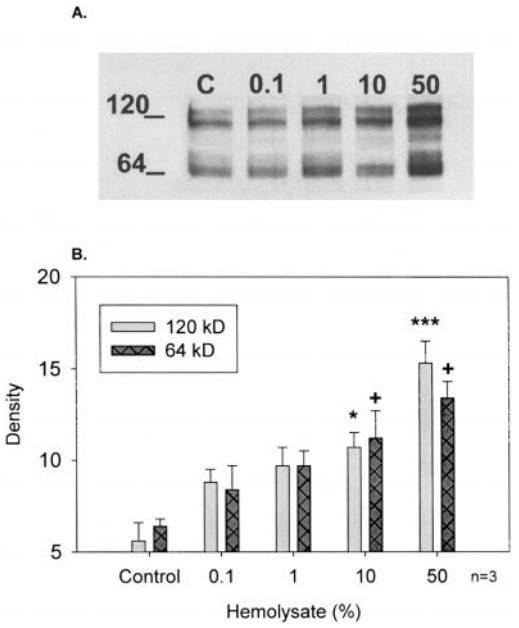

FIG. 2. Concentration dependency of hemolysate-induced tyrosine phosphorylation in cultured human dermal fibroblasts. Western blot stained with monoclonal antibody to phosphotyrosine. (A) Hemolysate was added to cells for the indicated concentrations (0.1, 1, 10, and 50%) for 5 min. Equal amounts of protein were loaded into each lane. Hemolysate enhanced tyrosine phosphorylation of the 120 and 64 kDa proteins in a concentration-dependent fashion. Tyrosine phosphorylation was enhanced by 1 and 10% hemolysate and the maximum effect was achieved by 50% hemolysate. (B) Summary of 3 experiments using human dermal cells. Data were quantified by a densitometer and the laser density was calculated and plotted. * and *** indicate $P < 0.05$ and 0.001 for 120 kDa. + indicates $P < 0.05$ for 64 kDa (ANOVA). "C" indicates control (without hemolysate), 0.1, 1, 10, and 50 are the concentrations of hemolysate (%).

Role of Fibroblasts in Cerebral Vasospasm

It was demonstrated that fibroblasts possess the ability to generate contractile forces (5, 6). These contractile forces may be accomplished through exertion of traction force on extracellular matrix in addition to simple contractility of cells themselves (5). This provides the possibility of attributing at least some portion of the change in tissue dimensions during vascular constriction to a reorganization, or packing, of extracellular matrix by these cells. Several investigators (21, 22) found proliferation of myointimal cells accompanied by increased deposition of collagen in the cerebral arteries after SAH. Smith *et al.* (21) used immunofluorescence techniques to detect collagen type V in a vasospastic cerebral artery 16 days after SAH. This was taken as evidence for proliferation of myofibroblasts within tunica media and intima during vasospasm, as these cells are known to synthesize collagen. Yamamoto *et al.* (25) reported that cerebrospinal fluid from SAH patients enhanced fibroblast populated collagen-lattice compaction. The degree of enhancement had a significant correlation with the grade of cerebral vasospasm after SAH. In another study, Yamamoto *et al.* (26) reported the capacity of myofibroblasts isolated from human cerebral arteries of vaso-

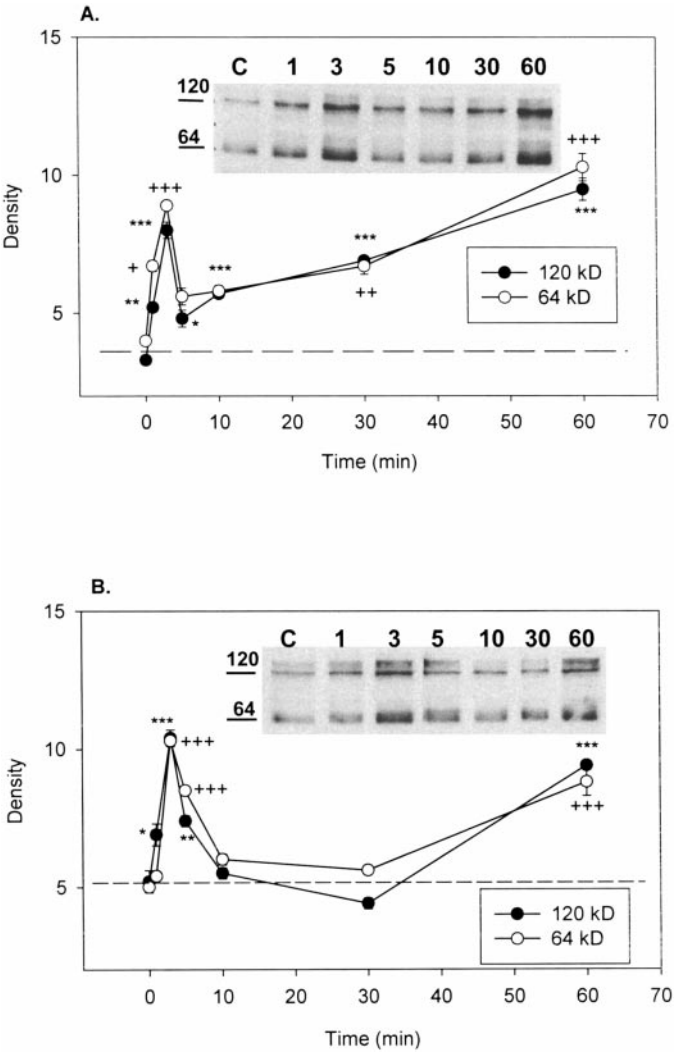

FIG. 3. Time dependency of oxyhemoglobin- and ATP-induced tyrosine phosphorylation in human dermal cells. (A) Three experiments using human dermal cells were summarized. Data were quantified by a densitometer and the laser density was calculated and plotted. Oxyhemoglobin (10 μM) was added to cells for the indicated times. Equal amounts of protein were loaded into each lane. Oxyhemoglobin enhanced tyrosine phosphorylation of the 120 and 64 kDa proteins in a time-dependent fashion. The response started at 1 min, peaked at 3 min, and decayed at 5 min. The response was enhanced again at 30 min and reached maximum at 60 min. The inset is a Western blot study showing oxyhemoglobin enhanced tyrosine phosphorylation at different times. (B) Three experiments using human dermal cells were summarized. Data were quantified by a densitometer and the laser density was calculated and plotted. ATP (10 μM) was added to cells for the indicated times and produced a biphasic tyrosine phosphorylation. The response started at 1 min, peaked at 3 min, and decayed to the resting level at 10 min. A second phase of tyrosine phosphorylation was observed at 60 min. The inset is a Western blot study showing ATP enhanced tyrosine phosphorylation at different times. *, **, and *** indicate $P < 0.05$–0.01–0.001 for 120 kDa and +, ++, and +++ indicate $P < 0.05$–0.01–0.001 for 64 kDa (ANOVA).

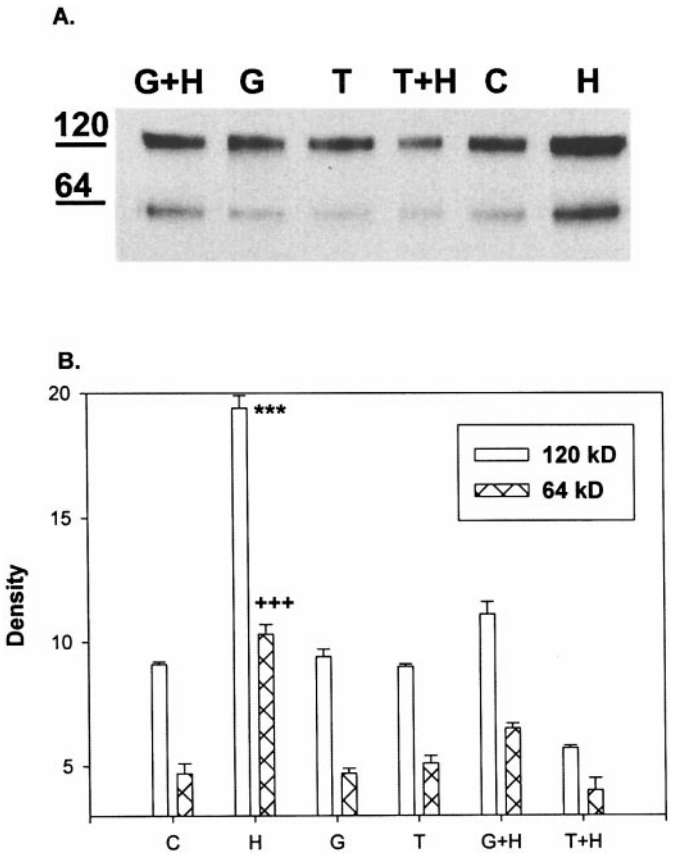

FIG. 4. Effect of tyrosine kinase inhibitors. (A) Hemolysate (H, 10%) was added to canine basilar arterial fibroblasts for 5 min in the presence of tyrosine kinase inhibitors genistein (G + H, 30 μM) and tyrphostin A51 (T + H, 30 μM). Saline (C) was used in the control group. Genistein (G) and tyrphostin A51 (T) were used without hemolysate in two groups. Hemolysate enhanced tyrosine phosphorylation of the 120 and 64 kDa proteins and the effect of hemolysate was abolished by genistein and tyrphostin A51. (B) Three experiments using canine basilar arterial cells were summarized. Data were quantified by a densitometer and the laser density was calculated and plotted. *** and +++ indicate $P < 0.001$ for 120 and 64 kDa, respectively (ANOVA).

spasm patient to compact the collagen lattice. This suggests that myofibroblasts in human cerebral arteries differ from medial smooth muscle cells and can generate a force rearranging the proliferated collagen matrix and this reorganization can contribute to, or be responsible for, sustained vasoconstriction. Studies by other investigators (10) evaluated the role of polypeptide growth factors released from intraluminal accumulated platelets. This may play an important role in arterial constriction caused by non-muscle components after SAH, by single or multiple mechanisms.

The possible role for fibroblasts in cerebral vasospasm was studied *in vitro* in both dermal and cerebral fibroblasts. Human dermal fibroblast cells were used and found to play an active role in collagen-compaction-induced bloody cerebrospinal fluid of vasospasm patients (22, 25). A similar fibroblast-collagen compaction induced by bloody cerebrospinal fluid was later reported in cultured human cerebral vascular fibroblasts (10, 26). Both dermal and cerebral fibroblasts were used recently in the investigation of the possible role of protein kinase C in the fibroblast-collagen compaction (19). Several reasons are responsible for switching cell types between cerebral and dermal and these reasons offered some justification for using dermal cells in some studies in this report. (a) Isolation, identification, and culturing cerebral vascular fibroblasts are extremely difficult. It was not unusual that some of the cerebral cells used before were

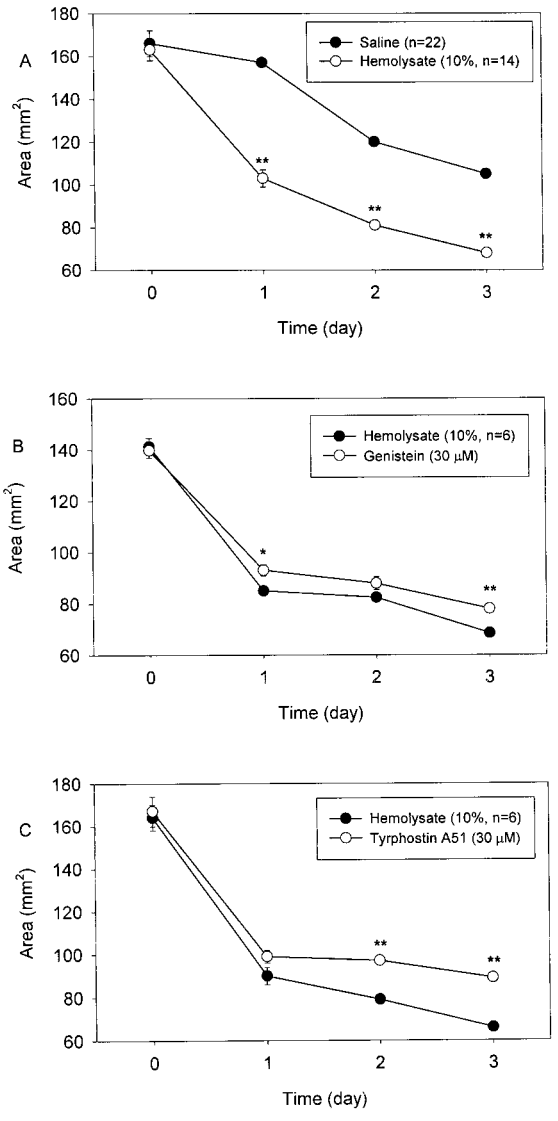

FIG. 5. Effect of tyrosine kinase inhibitors on FPCL compaction. (A) Hemolysate (10%) produced significant increase of FPCL compaction comparing with saline treated controls over 3-day periods in five experiments. (B) Incubation of FPCL with genistein (30 μM) slightly, but significantly attenuated the effect of hemolysate on day 1 and day 3 in five experiments. (C) Tyrphostin A51 (30 μM) reduced partially the effect of hemolysate on day 2 and day 3 in five experiments. * and ** indicate $P < 0.05–0.01$, respectively (ANOVA).

positive to the smooth muscle α-actin. Even though it was not clear if the cerebral fibroblasts were contaminated with smooth muscle cells, due to the lack of external lamina in cerebral artery (27), it is difficult to exclude this possibility. (b) Since the cell types were switched several times during the past years, it has been actually established that the dermal cells behaved similarly to those of cerebral fibroblasts (10, 19, 25, 26). (c) Furthermore, since the dermal cells are commercially available and are of much better quality (such as cell shape and growth rate), the data obtained are more solid, reliable, and comparable with those reported by others. (d) However, to avoid the possible underrepresentative of dermal cells for a cerebral problem, all key experiments were repeated in cultured canine basilar artery fibroblasts in this study. (e) Human cerebral fibroblasts were not used in this study due to the difficulties of safely and repeatedly obtaining tissues from patients.

Role of Tyrosine Kinases in Collagen-Lattice Compaction

Previous studies have demonstrated that bloody cerebrospinal fluid and growth factors increased fibroblast collagen-lattice compaction (10). However, the mechanisms of spasmogen-induced compaction have not been clearly documented. Protein phosphorylation has emerged, in recent years, as one of the key mechanisms for regulating signal transduction in eukaryotic cells. Transmembrane signaling through many receptor systems involves activation of a protein tyrosine kinase that is either an intrinsic part of the receptor or a protein that associates with it. Tyrosine phosphorylation of the receptor and/or substrates induces a cascade of protein interactions and enzyme activation through multiple pathways that lead to cellular responses such as transcriptional activation and cell proliferation (1, 23). Tyrosine phosphorylation of proteins causes various kinds of functional changes in the proteins that may be involved in pathological states and in physiological responses. Tyrosine kinases have shown to be involved in the contraction of peripheral smooth muscle either by activation of receptors or by opening of Ca^{2+} channels (1). Tyrosine kinases consist of three general subclasses: (a) membrane receptor tyrosine kinases, including the insulin receptor and receptors for epidermal growth factor and platelet derived growth factor; (b) cytosolic nonreceptor protein tyrosine kinases such as protooncogene products *Abl* and *Fes;* (c) membrane-associated nonreceptor tyrosine kinases related closely to $pp60^{v\text{-}src}$ (8). A large number of potential substrates for these tyrosine kinases, all believed to be directly involved in cell signaling, have been identified, including IP_3 receptors, phospholipase Cγ, and MAP kinase (8). However, little is known about their role in fibroblast compaction.

The roles of tyrosine kinases in endothelial (16) and smooth muscle cells (9, 12) have been described in our previous studies. This report demonstrated for the first time that hemolysate activated tyrosine kinases and increased collagen-lattice compaction in canine cerebral and human dermal fibroblasts. The participation of tyrosine kinases in the effect of hemolysate in fibroblasts was further supported by the effect of tyrosine kinase inhibitors genistein and tyrphostin A51. The concentration of genistein and tyrphostin A51 (30 μmol/L) used in this study is in the same range as that reported previously for smooth muscle and endothelial cells and is comparable to that required for inhibition of tyrosine kinase (1). At such concentration, these agents selectively inhibit tyrosine kinases but are without effect against other kinases such as cyclic adenosine monophosphate-dependent protein kinase (PKA) or protein kinase C (PKC) (1). Genistein, a natural tyrosine kinase inhibitor isolated from fungal extracts, is a competitive inhibitor of ATP in the kinase reaction and is noncompetitive with the protein substrate. Tyrphostins are synthetic inhibitors that possess the benzylidene moiety of erbstatin, a competitive inhibitor of protein substrate as well as ATP. In this study, genistein and tyrphostin A51, two structurally different inhibitors, reduced the effect of hemolysate, indicating that the effect of genistein and tyrphostin A51 was probably not non-specific. The inhibitory effect of tyrphostin A51 in hemolysate induced tyrosine phosphorylation and lattice compaction was more pronounced when compared to genistein may indicate that different tyrosine kinases or isotypes were activated by hemolysate in the arteries. The differential effects between tyrphostin A51 and genistein are not unexpected since there are many forms of tyrosine kinases in cells and inhibitors such as tyrphostins have shown considerable differences in potency in their action against different tyrosine kinases or different isotypes (1). For example, genistein markedly reduced L-type Ca^{2+} current in rat myometrial cells while tyrphostin A51 did not effect this current at all (14).

Mechanism of Hemolysate-Induced Tyrosine Phosphorylation

It has been established that the etiology of cerebral vasospasm is subarachnoid blood clots. Spasmogenic substances released from subarachnoid blood clots include oxyhemoglobin and ATP (28). Hemolysate has been shown to increase $[Ca^{+2}]_i$ in cerebral smooth muscle cells (28) and cerebral endothelial cells (29) and to produce contraction of cerebral arteries (20). The effect of hemolysate on $[Ca^{+2}]_i$ mobilization has been suggested to be mediated, at least partly, by tyrosine kinase phosphorylation (9). Hemolysate produced a dose-dependent increase in the level of tyrosine phosphorylation of two proteins approximately 70 and 110

kDa in cultured smooth muscle cells (9) and in cultured bovine endothelial cells (16). In this study, hemolysate produced a rapid and prolonged increase of tyrosine phosphorylation of two proteins, approximately 64 and 120 kDa, in canine basilar artery and human dermal fibroblast cells.

Oxyhemoglobin and ATP each produced smaller scaled but same bands of tyrosine phosphorylation in human dermal and canine basilar artery fibroblast cells. However, the time courses of tyrosine phosphorylation induced by oxyhemoglobin, ATP, and hemolysate are different. The tyrosine phosphorylation to hemolysate peaked at 5 min, then maintained at an elevated level to 60 min. On the contrary, the tyrosine phosphorylation to oxyhemoglobin and ATP peaked at 3 min and decayed at 5 min (even though they are still significantly higher than the resting level). The response to oxyhemoglobin picked up again at 30 min and reached the highest level, in the time studied, at 60 min. The response to ATP, on the other hand, decayed to the resting level at 10–30 min and re-peaked again at 60 min. Combining the tyrosine phosphorylation responses of oxyhemoglobin and ATP, superficially, will add up to a scale similar to that of hemolysate. However, the time course of response to oxyhemoglobin and ATP shifted 2 min to the left of that of hemolysate. Even though both oxyhemoglobin and ATP produced tyrosine phosphorylation of the same bands of proteins, their effect failed to mimic the effect of hemolysate in a precise manner.

The mechanism of hemolysate-induced tyrosine phosphorylation and lattice compaction is not clear. There are several possibilities. (a) Activation of G-protein-coupled receptors causes either hydrolysis of inositol phospholipids by phospholipase C or activation of tyrosine kinase, possibly by the $\beta\gamma$ subunits of heterotrimeric G proteins (1, 8). Therefore, activation of G-protein-coupled receptors may activate tyrosine kinases. Our previous data have shown that ATP by binding with P_2 receptors (a G-protein-coupled receptor) increases intracellular Ca^{2+} and contracts cerebral arteries (20, 28). However, even though ATP induced tyrosine phosphorylation of the same two proteins, the time course of phosphorylation response to ATP was different from that of hemolysate. (b) Oxyhemoglobin was suggested to activate tyrosine kinases (24) and may be involved in the effect of hemolysate since hemolysate contained oxyhemoglobin in this study. However, oxyhemoglobin failed to mimic the time course of tyrosine response to hemolysate. (c) In speculation, other molecules in hemolysate may contribute to tyrosine phosphorylation and a mixture of these molecules may re-shape the time course of tyrosine response.

Conclusions

We conclude that hemolysate enhances tyrosine phosphorylation in canine cerebral and human dermal fibroblast cells. The effects of hemolysate were mediated by oxyhemoglobin, ATP, and other unknown molecules. Elevated tyrosine phosphorylation may play a role in collagen compaction. The tyrosine kinase pathway may be involved not only in the contraction of smooth muscle cells but also in the compaction of fibroblasts. Thus, tyrosine kinases may be important in the pathogenesis of cerebral vasospasm and tyrosine kinase inhibitors may be useful in the management of cerebral vasospasm.

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