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Poloxamer 188 Enhances Functional Recovery of Lethally Heat-Shocked Fibroblasts

F. A. Merchant, Ph.D., W. H. Holmes, B.S., M. Capelli-Schellpfeffer, M.D.,*
R. C. Lee, M.D., Ph.D.,* and M. Toner, Ph.D.[1]

*Center for Engineering in Medicine and Surgical Services, Massachusetts General Hospital; Harvard Medical School, and Shriners Hospitals for Children, Boston, Massachusetts 02114; and *The University of Chicago, Department of Surgery, Chicago, Illinois 60637*

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Damage to the cell membrane has been implicated as the primary event in the pathogenesis of heat shock, generally resulting in loss of cellular homeostasis and cell death. Thus a promising mode of therapy would involve the restoration of cell membrane integrity. Surfactant molecules, specifically triblock polymers such as Poloxamer 188 (P-188), possess the ability to self-aggregate into membrane-like structures in aqueous solutions and have been shown to restore membrane integrity. The objective of this study was to develop functional and morphological assays to determine whether treatment with P-188 after heat shock enhances the recovery of thermally damaged cells. Human foreskin fibroblasts were placed in sterile vials and heated by immersion in a calibrated water bath for various lengths of time at predefined temperatures. Cell recovery after heat shock was assessed using a functional assay based on the ability of the cells to contract fibroblast populated collagen lattices (FPCLs). Subsequent to heating, collagen lattices were prepared with control (no heat, no P-188) and heat shocked cells (with and without P-188). Our results indicate that treatment with low concentrations of P-188 after heat shock was effective in ameliorating both the morphological integrity and the contractile function of thermally damaged cells. Further, we observed that P-188 was most effective in improving the contractile ability of cells heat shocked at 45°C; however, it had no influence on the contractility of cells exposed to higher temperatures. Our results suggest that there exists a threshold of thermal stress (45°C for 20–60 min) beyond which treatment with low concentrations of P-188 (0.5 mg/ml) is ineffective in minimizing cell damage. Moreover, the results of our morphological assays indicate that cells treated with P-188 after heat shock maintain their cytoskeletal organization, whereas untreated cells exhibit filamentous actin depolymerization. © 1998 Academic Press

[1] To whom all correspondence should be addressed at Shriners Burns Institute Research Center, One Kendall Square, Bldg. 1400 W, Cambridge, MA 02139. E-mail: mtoner@sbi.org. Fax: (617) 374-5665.

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INTRODUCTION

Heat shock in mammalian cells and tissues can occur as a result of surface heating during thermal burns or via deep tissue heating during electrical trauma. In both cases, some of the early events include a direct effect of heat on cells and tissues [1]. Heating has been known to induce a plethora of morphological and physiological changes in cells and tissues that eventually lead to cell death and necrosis [2–4]. Some of the cellular components that are susceptible to heat injury include the plasma membrane, cellular proteins and enzymes, cortical microfilaments and microtubular proteins of the cytoskeleton, subcellular organelles, polyribosomes, nucleoplasm and nucleoli, and DNA [5–9]. Although, many of these alterations result in heat shock-induced cell death, debate still persists with regard to the actual sequence, the extent of reversible or irreversible events, and the degree of damage that can be tolerated by the cell to survive.

Damage to the cell membrane has been shown to result in loss of cellular homeostasis and cell death, and is thus implicated as a critical event in the pathogenesis of heat shock [5–7]. Plasma membrane modifications including membrane blebbing and surface invaginations are the most common early morphological alterations observed in heat shocked cells [6, 10]. These primary lesions typically lead to a cascade of secondary events that can prove to be fatal. Heating modifies surface-membrane interactions such as cell adhesion, affecting the ability of cells to attach and crawl, thus resulting in decreased function and proliferation [11]. Furthermore, plasma membrane binding function is are also affected by heat shock [2, 7, 12]. Similarly, plasma membrane permeability is also compromised when cells are subjected to heat shock [5]. Changes in the transport properties of the plasma membrane result in an imbalance in the intracellular ionic environ-

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ment [13], causing a decrease in membrane surface charge and leading to plasma membrane depolarization [14]. Further, an increase in membrane permeability typically results in the consumption of high energy compounds like adenosine triphosphate (ATP), having diverse secondary effects on metabolism and growth. The plasma membrane thus plays an important role in governing heat shock-induced damage and cell lysis, and loss of cell membrane integrity is an important factor in the pathogenesis of cell and tissue injury as seen in victims of both electric shock and thermal burns [1].

Thus it seems that strategies to stabilize the structural integrity of cell membranes, may help promote the salvation of thermally injured cells and tissues. A biologically safe method for achieving this would involve the use of biocompatible materials that mimic the properties and composition of cell plasma membranes. Several studies have explored the therapeutic potential of artificial surfactants, specifically the poloxamer series of nonionic coblock polymers, as biologically safe materials that act as membrane sealants [15–17]. These compounds have been shown to spontaneously aggregate in aqueous solutions to form membrane bilayers and to preferentially adhere to cellular plasma membranes [18].

Poloxamer 188 (P-188) is one of the most widely used artificial surfactants and is currently being tested in treatment of burn wound injuries, as well as other dermatological and surgical disorders [15–18]. It also has numerous therapeutic uses as a drug delivery system, topical formulation, and an antithrombolytic agent [19–24]. Moreover, P-188 has been approved by the Food and Drug Administration for use in humans, and several clinical studies have been completed in patients using an aqueous solution of P-188 formulated for intravenous administration [19, 23]. In a recently reported 114 patient pilot thrombolytic trial, adjunctive therapy with P-188 markedly reduced myocardial infarct size, increased myocardial salvage, increased the residual left ventricular ejection fraction, and reduced the incidence of in-hospital reinfarction [24]. Most importantly, P-188 has low toxicity (provided the dose is adjusted for renal function), has a half-life of ~5 h after intravenous injection, is excreted in the urine unmetabolized, and has been demonstrated to be well tolerated in clinical studies on human volunteers [25].

More recently, studies on biological and artificial membranes demonstrated the potential of P-188 to effectively stabilize damaged membranes [15, 16, 26–28]. Sharma *et al.* [27] have shown that P-188 interacts with artificial lipid membranes and renders them more resistant to electroporation. In other studies, Lee *et al.* [15, 26] have used *in vitro* (skeletal muscle cells) and *in vivo* (rat muscle flap) models to demonstrate the effects of P-188 on electrically injured biological membranes. They observed that treatment with P-188 after electroporation seals membrane pores and substantially reduces tissue injury. In previous studies, our laboratory demonstrated that plasma membrane integrity is continuously compromised while cells are subjected to elevated temperatures [5], and showed that P-188 is moderately effective in sealing thermally induced membrane defects [16]. However, its effect on cell function and survival is yet unknown.

In this study, we present morphological and functional assays to determine whether treatment with P-188 after heat shock enhances the recovery of thermally damaged cells. To this end, we have used the contraction of fibroblasts populated collagen lattices (FPCLs) as a functional assay to assess the efficacy of P-188 in reducing thermal damage to cells. Fibroblasts seeded in collagen lattices exhibit the ability to reorganize the gels resulting in lattice contraction [29]. It has been proposed that the FPCLs change in size either as a result of traction forces generated by cell locomotion or as a result of the isometric tension applied by the cells on collagen fibrils [30]. Therefore, collagen gel contraction is a reasonable functional indicator of normal cell contractile function. Results from this assay indicate that there is a threshold of thermal stress for which treatment with low concentrations of P-188 is effective in minimizing cell damage, and that P-188 treated cells maintain their cytoskeletal integrity.

MATERIALS AND METHODS

Cell culture and heat shock. Human foreskin fibroblasts were obtained using a primary explant technique and stored in liquid nitrogen after slow freezing [31]. A cell line was initiated for culture by thawing a vial of cells and resuspending the cells in culture medium after centrifugation at 800 rpm for 5 min. Cells were plated in 75-cm^2 tissue culture flasks (Becton Dickinson & Co., Franklin Lakes, NJ, No. 3024) using 12 ml of Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO, No. D-5030) with 10% fetal bovine serum (FBS, Sigma, No. F-2442), 2% penicillin-streptomycin (PS, Sigma, No. G-1146) and kept in a humidified (10% CO_2 and 95% air) incubator at 37°C. At confluency the cells were passed using trypsin (0.05% for 5 min at 37°C, Sigma, No. T-8802) and plated out 1:5 in flasks. Cultures were discarded before the 15th passage and a new cell line was initiated.

For heat shock experiments, the cells were heated in suspension after trypsinization. The cells were resuspended in culture medium and 2-ml suspensions containing 2×10^5 cells/ml were placed in sterile cryogenic vials (Nalge Company, Rochester, NY, No. 5000-0020) and heated by immersion in a calibrated water bath for various time periods at predefined temperatures (~10 min elapsed between the addition of trypsin and immersion of cells in the water bath). Typically, the cells were subjected to a temperature within ±0.2°C of the desired temperature for at least 90% of the exposure time (the cell suspension reached the desired temperature in <3 min). The temperature of the medium in the vial was found to agree precisely with that of the water bath. Control cells were placed in sterile vials identical to those used for heating experiments and incubated at 37°C for equivalent time periods.

Functional assays using FPCLs. For the functional assays, collagen stock solution was prepared using Type IV rat tail collagen at a density of 0.5–0.6 mg/ml, by rapidly mixing 9 ml of collagen with 1 ml of 10 × DMEM [32]. To prepare lattices, 1 ml of the cell suspension was mixed with 1 ml of collagen stock and pipetted into six-well, nontreated tissue culture plates (Corning Costar, Cambridge, MA, No. 9088). The lattices were allowed to gel for ~2 h at 37°C, after which they were released to produce free-floating lattices and 1 ml of culture media was added to each well. The plates containing free-floating lattices were then returned to humidified (10% CO_2 and 95% air) tissue culture incubators at 37°C. At predefined time points during the experiment, lattice diameters were measured by placing

the dishes on a transparent metric scale and taking the average of the major and minor axes as the diameter.

For evaluating the influence of P-188 on heat shocked cells, FPCLs were prepared for three different conditions: control (no heat shock, no P-188), heat shock (heat shock, no P-188), and P-188 (heat shock, P-188). Following heating, two sets of tubes for the heat shocked cells (four to six tubes in each set) and one set of tubes for the control cells each containing 0.5 ml (10^5 cells) of the cell suspension were prepared. One set of tubes containing the heat shocked cells received 0.5 ml of P-188 (BASF Corporation, Wyandotte, MI, No. WPEO 556B) at 4× the desired concentration in media, and the other set received 0.5 ml of culture medium. Similarly, the set of tubes containing control cells received 0.5 ml of culture medium. FPCLs were obtained (as described earlier) and placed in humidified (10% CO_2 and 95% air) tissue culture incubators at 37°C. The rate of initial contraction of the free-floating collagen gels was determined by measuring the diameter of the circular gels at 0 and 24 h after seeding.

Further, for FPCL assays using P-188, experiments were conducted to determine a method to treat the cells in lattices with P-188. We tested three different conditions: incorporating P-188 in the lattices, adding P-188 to the media in the dishes, and using P-188 in the lattices plus media. The procedure for preparing the lattices has been described earlier. We also prepared lattices using control cells (no heat) with P-188 to determine if P-188 had any effect on the ability of normal cells to contract collagen lattices.

In addition, experiments were also performed to determine the influence of the number of cells on lattice contraction. In order to determine if cell proliferation occurred in the lattices over the duration of the experiment (24 h), individual lattices were digested using collagenase IV (Sigma, No. C-5138). Briefly, at 24 h after seeding, the medium was removed from the free-floating lattices and 0.1% collagenase in Krebs-Henseleit bicarbonate buffer (KHB, Sigma, No. K-3753) was added. The collagenase-treated lattices were incubated at 37°C for 20–30 min. At this time, 1 ml of ice-cold KHB was added to the dishes to stop the digestion. The lattices dissolved completely leaving behind cells in suspension. The collagenase was removed by centrifugation and the cells were resuspended in KHB and counted using a hemocytometer.

Determination of cytoskeletal integrity. Since the cytoskeletal network plays a crucial role in the process of lattice contraction, we evaluated the structural integrity of the heat shocked cells treated with and without P-188 via confocal microscopy of the cytoskeletal morphology. Cytoskeletal integrity was determined via fluorescence imaging of the rhodamine phalloidan (Molecular Probes, Eugene, OR, No. R-415) stained filamentous actin network. FPCLs were prepared as described earlier for the three conditions of control, heat shock, and P-188, with the exception of including glass coverslips in the wells to facilitate sample preparation for confocal microscopy. Following 24 h of incubation at 37°C, the medium was removed and the lattices were washed three times with phosphate-buffered saline (PBS, Biofluids Inc., Rockville, MD, No. 313) and fixed with 2% paraformaldehyde (Fisher, Springfield, NJ, No. T-353) in PBS for 20 min. After three washings with PBS, a 1:100 dilution of rhodamine phalloidan in PBS was added, and the lattices were incubated for 60 min at 37°C. Thereafter, the cells were washed and the coverslips holding the lattices were mounted on slides with an *n*-propyl galate (Sigma, No. P-3130)/glycerol (Sigma, No. G-8773) mounting solution as an antifade agent. Images of the cytoskeletal architecture were acquired using a laser scanning confocal microscope (BioRad, Hercules, CA, MRC 600). The images were qualitatively assessed to evaluate the cytoskeletal morphology.

Data analysis and statistics. All the experiments involving the FPCLs were performed at least three times for the individual heat shock conditions, with an average of five lattices per run. Similarly, for evaluation of the cytoskeletal integrity, experiments were performed three times for the individual heat shock conditions and at least two slides were prepared and imaged for each condition. Error bars represent the standard error of the mean, and statistical analysis was performed using the Student's *t* test (Microsoft Excel, Seattle, WA).

RESULTS

Description of Functional Assays Using FPCLs

Based on prior work from our laboratory [4], we used a heat shock protocol of 45°C for 20 min to determine the effect of thermal damage on the contractile function of cells. Human foreskin fibroblasts were subjected to heat shock, after which the cells were treated with P-188 or left untreated, and subsequently incorporated into collagen lattices. Control lattices were prepared from unheated cells that had been incubated at 37°C for 20 min. Figure 1 depicts a micrograph from a typical experiment. It is evident that following heat shock the cells appear to have a reduced ability to contract gels. On the other hand, when cells were treated with a 0.5 mg/ml concentration of P-188 after heat shock at 45°C for 20 min the ability of the heat shock cells to contract collagen lattices during the next 24 hours was improved.

Next, we quantified the diameter of FPCLs as a function of time. Figure 2 presents lattice contraction from a typical experiment over a period of 6 days for control (unheated) cells, controls cells treated with 0.5 mg/ml of P-188, heat shocked cells (45°C, 20 min), and heat shocked cells (45°C, 20 min) treated with 0.5 mg/ml of P-188 after the heat shock. As can be seen in the representative experiment depicted in Fig. 2, control (unheated) cells, control (treated with P-188), and heated cells treated with P-188 behave qualitatively in a similar manner; whereas cells heated and not treated with P-188 display reduced ability to contrast lattices. It should be noted that although the amount of contraction in the lattices differs over the first 24–48 h, at 6 days the lattice diameters appear to reach a common minimum value. This phenomenon of achieving a minimum diameter is common to the mechanism of lattice contraction by fibroblasts as reported by other investigators [32]. Another, common finding which is also evident in Fig. 2 is that maximal contraction is observed in the lattices in the first 24–48 h following seeding [33]. Hence in all following experiments, we monitored the rate of contraction of the lattices at 24 h as a quantitative measure of the contractile ability of the fibroblasts for statistical comparisons.

Effect of P-188 Concentration on the Contractile Ability of Heat Shocked Cells

We performed experiments to monitor the effect of different concentrations of P-188 on the contractile ability of heat shocked cells. Cells were treated with six different concentrations of P-188 (0.1, 0.25, 0.5, 2, 4, and 10 mg/ml) following a heat shock of 45°C for 20 min. The initial rate of contraction of the heat shocked cells was normalized with respect to that of unheated controls from the same experiment. This normalization was necessary because the contraction of the control lattices varied with different runs due to inherent biological factors as well as difficulties in precisely controlling various experimental parameters (e.g., percentage confluency at trypsinization of cells and the seeding

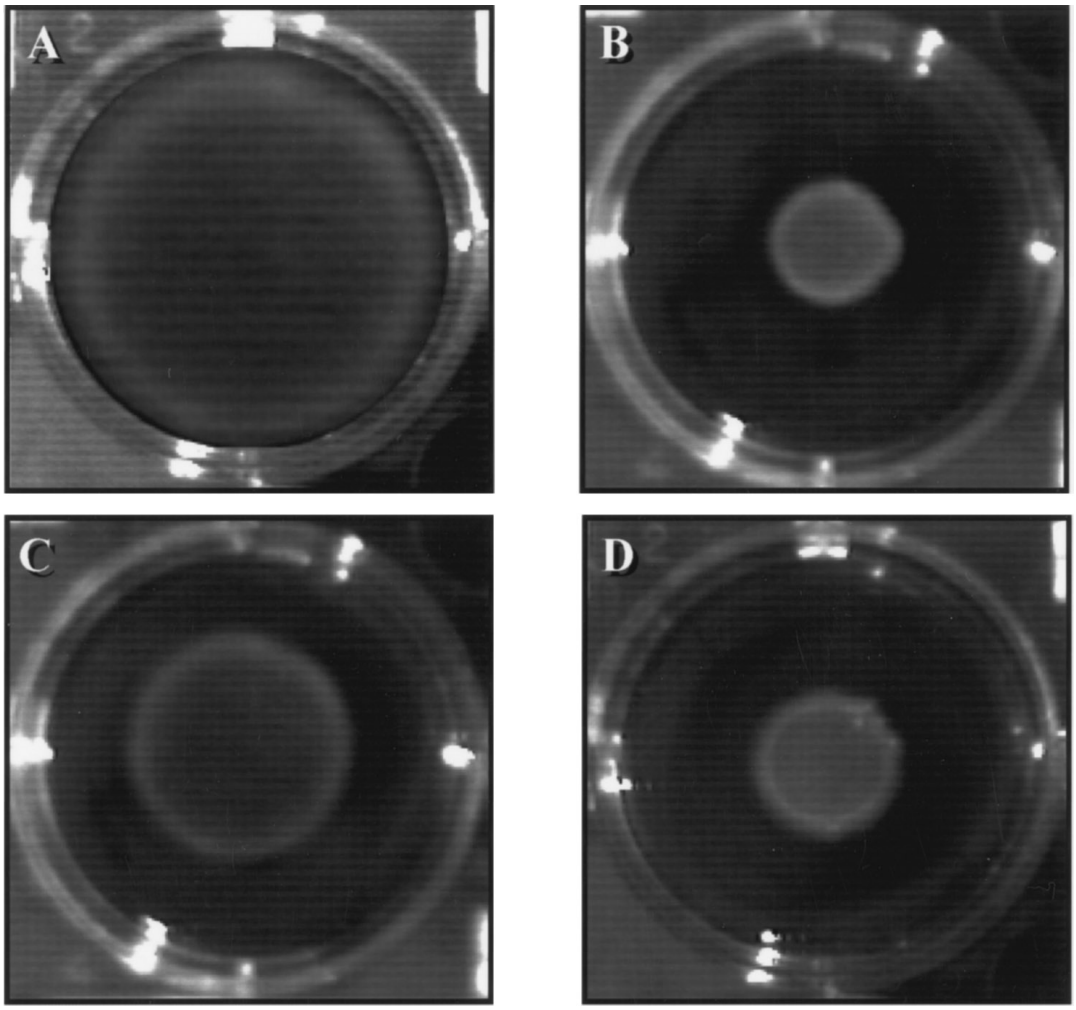


FIG. 1. Micrographs illustrating lattice contraction in FPCLs by (A) unheated controls at 3 h after seeding, (B) unheated controls at 24 h after seeding, (C) cells exposed to heat shock at 45°C for 20 min, and (D) cells treated with P-188 (0.5 mg/ml) after heat shock (45°C, 20 min).

density of cells) As shown in Fig. 3, these results indicate that low concentrations of P-188 ($\leq$ 0.5 mg/ml) are most effective in improving the ability of heat shocked cells to contract collagen gels (using a null hypotheses for equal means; for untreated vs. P-188 treated, P = 0.0003, 0.0007, and 0.004 for 0.1, 0.25, and 0.5 mg/ml, respectively). Although a P-188 concentration of 2 mg/ml has a significant effect on lattice contraction in the heat shocked cells (P = 0.004), the improvement in the rate of contraction is less, relative to a P-188 concentration of 0.5 mg/ml. Concentrations of P-188 $>$2 mg/ml do not seem to have a significant beneficial effect on the heat shocked cells (P = 0.307 for 4 mg/ml, and P = 0.339 for 10 mg/ml). It should be noted that there is some variation in the rate of contraction of the untreated heat shocked cells for the six data sets presented in Fig. 3. This is because the experiments with the different concentrations of P-188 were performed at different times, and not in the same experiment. Furthermore, the contraction of the FPCLs is influenced by several factors including the concentration of serum in the culture medium and collagen density. Although, precautions were taken to maintain equivalent concentrations of both in all the experiments performed, it is possible that slight variations may be present in the same due to operator bias, augmenting the variations observed in the contraction of the untreated cells. Finally, since heat injury in cells is a stochastic process, it is highly probable that differences in the contractile ability of the heated cells may be observed among the different experiments.

Next, we tested the effect of different modes of delivery of P-188, namely in media, in lattice, and in lattice plus media. Our results indicated that the mode of delivery did not significantly effect cell survival (Table 1), and hence we applied P-188 after heat shock in the lattices and media in all of the experiments performed. These experiments indicate that post-heat shock treatment with P-188 is nontoxic to human foreskin fibroblasts, and low concentrations of P-188 are effective in improving fibroblasts function after a mild heat shock.

Effect of P-188 on Cells Subjected to Varying Degrees of Heat Shock

Having observed an improvement in the contractile function of cells treated with low concentrations of P-

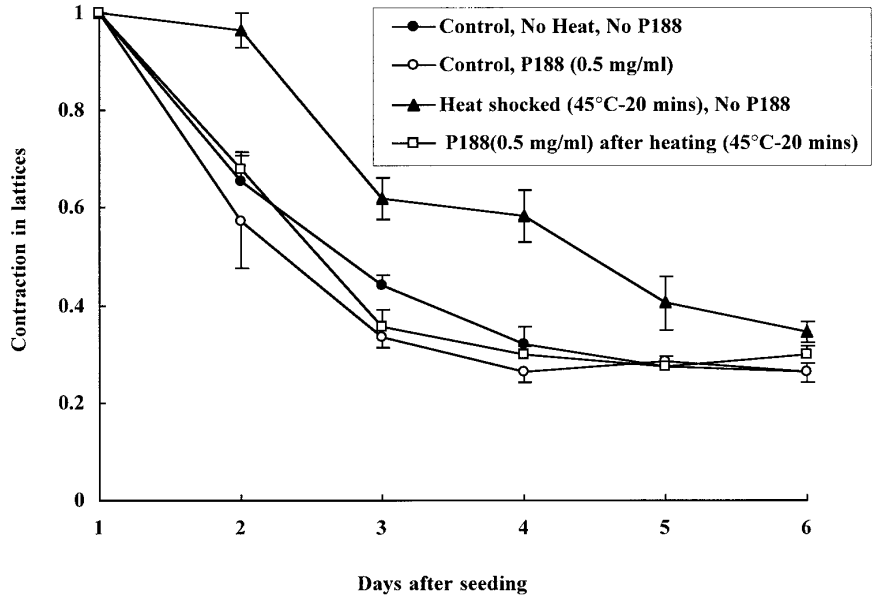


FIG. 2. Plot of contraction in FPCL over a period of 6 days from a typical experiment. The contraction in lattices for four different conditions is presented: control untreated cells, control cells treated with P-188, heat shocked cells (45°C for 20 min), and P-188 treated heat shocked cells (45°C for 20 min). Error bars represent the standard error of the mean from one experiment with five lattices.

188 after a heat shock of 45°C for 20 min, we next performed experiments to determine the extent of P-188's beneficial influence on thermally damaged cells. For a given rate of heat shock exposure, the probability that a cell will live or die is a function of the temperature and the duration of heat exposure [4, 34]. We performed a series of experiments to determine the severity of heat shock for which P-188 is capable of enhancing the contractile ability of the cells. Since, we found that low concentrations of P-188 were most effective in treating cells heat shocked at 45°C for 20 min (Fig. 3), we used a P-188 concentration of 0.5 mg/ml in this

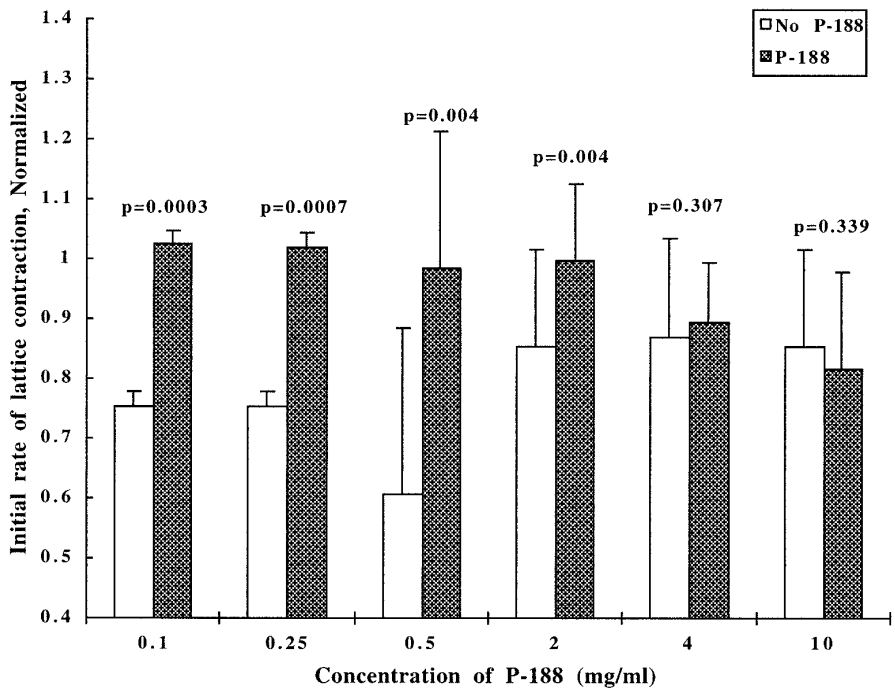


FIG. 3. Effect of treatment with different concentrations of P-188 on the rate of contraction of FPCLs. The fibroblasts were heat shocked at 45°C for 20 min and different concentrations of P-188 were applied to the cells after heat shock. The initial rate of contraction was determined by measuring the diameter of the lattices at 0 and 24 h after seeding. The rate of contraction in both untreated and P-188 treated cells is normalized with respect to that in unheated control cells. Error bars represent the standard error of the mean from triplicate experiments with five lattices per experiment.

TABLE 1

Effect of the Mode of Delivery of P-188 on Contraction of Lattices

Condition	No P-188	P-188 (0.5 mg/ml) mode of delivery		
		Media	Lattice	Lattice + media
Control	0.81 ± 0.01	0.79 ± 0.01	0.79 ± 0.01	0.84 ± 0.01
Heat shocked	0.34 ± 0.05	0.89 ± 0.01	0.89 ± 0.02	1.0 ± 0.05

Note. Cells were heat shocked at 45°C for 20 min and were seeded in lattices with or without P-188 (0.5 mg/ml) after the heat shock. Similarly, control cells (unheated) were incubated at 37°C for 20 min and seeded into lattices with and without P-188. P-188 was added to the lattices using three different modes of delivery: in lattice, in media, and in lattice plus media (see the methods section for a description of the procedure). The rate of contraction of the lattices was measured at 24 h after seeding.

experiment. The cells were exposed to three different temperatures 43, 45, and 48°C for 20, 40, and 60 min each, and treated with 0.5 mg/ml of P-188. The initial rate of contraction for heat shocked cells treated with and without P-188 was normalized with respect to unheated controls and is presented in Fig. 4. P-188 was most effective in improving the ability of cells to contract the collagen gels at 45°C. The initial rate of lattice contraction was statistically significantly improved in the presence of P-188 at 45°C for all three heat shock durations of 20, 40, and 60 min with *P* values of 0.04, 0.025, and 0.001, respectively. On the other hand, P-188 had no beneficial influence on the recovery of the contractile ability of cells exposed to at a higher temperature of 48°C. In fact, there was no measurable contraction of the FPCLs in both control and P-188 treated cells subjected to 48°C for all three time points; as a result, no statistical analysis could be performed. At 43°C, since the cells incur minimal damage, the beneficial effect of P-188 was less dramatic than at 45°C. For heat shock durations of 40 and 60 min, the P-188 had a statistically significant improvement in restoring the contraction of FPLCs with *P* values of 0.05 and 0.001, respectively. For 20 min of exposure to 43°C, there was no statistical significance between the P-188 and control (*P* value = 0.2) These results suggest that there exists a range of heat shock (45°C for 20–60 min) during which treatment with P-188 is effective in enhancing contractile ability in cells.

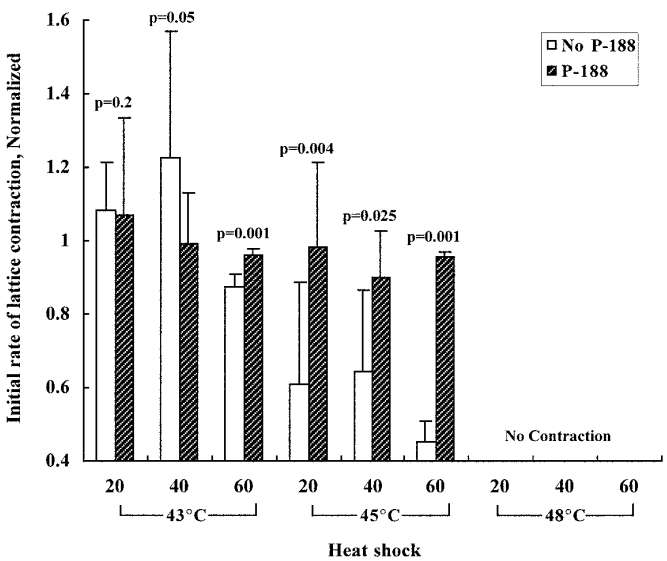


FIG. 4. Effect of P-188 (0.5 mg/ml) on fibroblasts subjected to varying degrees of heat shock. The cells were subjected to heat shock at three different temperatures (43, 45, and 48°C), for 20, 40, and 60 min. P-188 was applied to the cells after heat shock and the rate of contraction in the lattices was measured at 24 h after seeding. Error bars represent the standard error of the mean from triplicate experiments with five lattices per experiment.

In order to better elucidate this observation, we hypothesized that a probable cause for the inability of P-188 to improve the contractile ability of cells subjected to heat shock at higher temperatures (48°C) may involve a denaturation of the surfactant. The structural integrity of P-188 may be compromised due to the damaging action of free radicals that are liberated when cells are subjected to heat shock [35]. In order to test this hypothesis, we first exposed P-188 to cells subjected to heat shock at elevated temperatures and tested the efficacy of this heat reconditioned P-188 in treating cells subjected to a less severe heat shock. Cells heat shocked at 50°C for 60 min were incubated with P-188 for 30 min, and this reconditioned P-188 was then used to treat cells subjected to a heat shock at 45°C for 20 min. The reconditioned P-188 proved to be equally effective in improving the contractile ability of the heat shocked cells (normalized rate of contraction at 24 h, 0.96 ± 0.06) as the unprocessed P-188 (normalized rate of contraction at 24 h, 0.95 ± 0.1).

In order to better understand the differences in the rate of contraction of the P-188 treated and untreated heat shock cells, we evaluated the influence of cell number on lattice contraction. In order to determine that a change in the number of cells in the lattices was not responsible for the differences in the rate of lattice contraction observed between the P-188 treated and untreated cells after heat shock, we counted the number of cells in the lattices at 24 h after seeding. The collagen gels were digested using collagenase and the released fibroblasts were counted. There were no significant differences in the number of cells in both the P-188 treated ($1.15 \times 10^5 \pm 23{,}000$) and untreated lattices ($1.03 \times 10^5 \pm 17{,}000$) for the heat shocked cells. The number of cells initially incorporated in the lattices was ($1.15 \times 10^5 \pm 27{,}000$), and our data suggest that this number remains constant at 24 h after

seeding, indicating that the cells do not significantly proliferate during the duration of the experiment. These data correlate with other studies that have shown that the time of initial gel reorganization correlates with initial cell spreading, and that during the first 48 h cell proliferation has no effect on lattice contraction [33, 36]. In addition, the viability of fibroblasts exposed to 45°C for 20 min was also measured using trypan blue exclusion assay. There was no statistical significance between no P-188 (91 ± 5%) and P-188 (98 ± 4%) treatment conditions (P value = 0.0235). Because the cell number and viability in the lattices are relatively constant at 24 h, it may be concluded that the difference in the rates of contraction between the untreated and P-188 treated cells is influenced by the action of the surfactant on the cells.

Effect of P-188 on the Cytoskeletal Integrity of Heat Shocked Cells

In light of the fact that lattice contraction requires the active participation of the cytoskeleton, we performed experiments to determine whether treatment with P-188 after heat shock had any effect on the cytoskeletal integrity of the cells. We performed experiments at 45°C for 20, 40, and 60 min to elucidate the effect of P-188 on the cytoskeletal integrity of cells via fluorescent labeling of the filamentous actin network. Figure 5 presents micrographs of fluorescently labeled, control (unheated) cells, untreated heat shocked cells, and heat shocked cells treated with 0.5 mg/ml of P-188 (45°C for 20 min). As shown in Fig. 5A, the control (no heat, no P-188) cells were mainly of stellate form, with a round or oval cell body and long filopodial processes. Almost all the control cells showed the presence of an intact and normal filamentous actin network. On the other hand, most of the untreated heat shocked cells exhibited severe filamentous actin depolymerization, and a majority of the cells were strikingly different from the untreated controls, with a pear-shaped or rounded form and short filopodial processes (Fig. 5B). The organization of filamentous actin in most of the P-188 treated heat shocked cells (Fig. 5C) appeared to be similar to that of unheated controls with a close to normal cytoskeletal morphology. These results suggest that P-188 may play a role in the stabilization of the cytoskeletal network of heat shocked cells. Further, we observed that for the P-188 treated heat shocked cells, the number of cells displaying an intact cytoskeletal architecture (stellate form) were predominant, and very few rounded cells were present. These results suggest that P-188 may have a beneficial influence on a fraction of cells that have been damaged following heat shock.

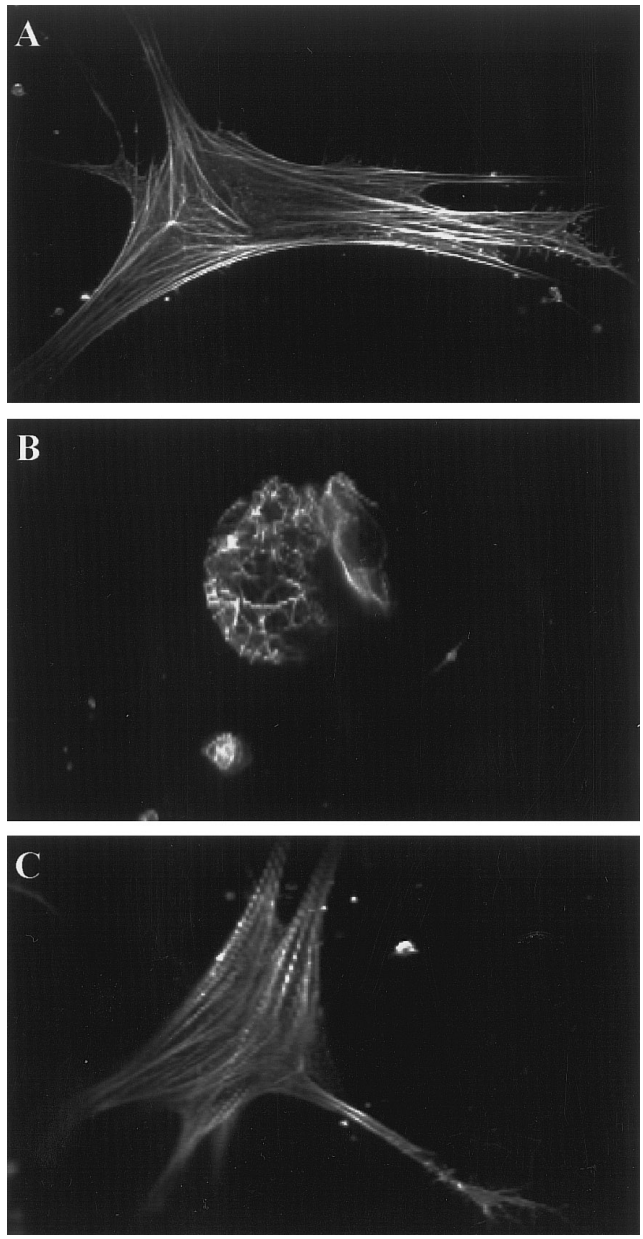


FIG. 5. Micrographs illustrating the effect of P-188 (0.5 mg/ml) on the cytoskeletal microfilaments of fibroblasts subjected to a heat shock of 45°C for 60 min. (A) Control (no heat, no P-188), (B) heat-treated (no P-188), and (C) heat-treated (P-188).

DISCUSSION

We have explored the potential of P-188 in ameliorating cell recovery after heat shock. Recent studies have shown that treatment of electrically and thermally injured cells with P-188 helps maintain the integrity of the cellular plasma membrane [1, 15, 16, 26]. The objective of this study was to determine whether P-188 has a positive influence on the functional recovery of heat shocked cells. To accomplish this, we employed a functional assay using FPCLs in conjunction with fluorescent labeling of the cytoskeletal microfilaments (filamentous actin) to evaluate the efficacy of treatment with P-188 after heat shock on cell recovery. Our data suggest that treatment with P-188, after heat shock, positively influences cell function with respect to the contractile ability of the cells and stabilization of the cytoskeletal infrastructure.

We initially demonstrate that heat shock modifies

the contractile function of fibroblasts. Following heat shock, the cells show a marked reduction in their ability to contract collagen gels (Figs. 1 and 2). These results correlate well with those of other researchers who have shown that cells with defects exhibit a decrease in their ability to contract collagen lattices [29, 36]. Further, it appears that P-188 has a beneficial influence on the contractile ability of cells for a certain threshold of heat shock (45°C for 20–60 min). In order to determine the extent of cell recovery induced by P-188 treatment, we compared the contraction of the untreated cells with that of P-188 treated cells exposed to 45°C for 20 min. From our results, we observed that a change in either the number or the viability of cells in the lattices was not responsible for the differences in the rate of contraction observed in the treated and untreated lattices.

Lee *et al.* [15, 26] and Padanilam *et al.* [16] have individually demonstrated the effectiveness of low concentrations of P-188 (below the CMC of 1 mg/ml at 25°C) in sealing damaged plasma membranes of skeletal muscle cells following electrical and thermal injury. Accordingly, our results show that treatment with low concentrations of P-188 ($<$0.5 mg/ml) after heat shock has a beneficial effect on the contractile ability of HFF, whereas higher concentrations of P-188 are less effective in enhancing the contractility of the heat shocked cells. A probable explanation may involve the existence of different physical states of P-188 at different concentrations above and below the CMC. Studies have shown that P-188 molecules exist as individual monomers at concentrations below the CMC, and as micellar aggregates at concentrations greater than the CMC. The differences in the interaction of the various physical forms of P-188 (monomers or micellar aggregates) with the cell membranes may be responsible for the variation in the effectiveness of different concentrations of P-188 in enhancing the contractile ability of the cells following heat shock.

An interesting finding of this study is that for cells exposed to varying degrees of heat shock there appears to be a threshold of heat stress (45°C for 20–60 min) for which treatment with P-188 is effective in enhancing cell recovery, and beyond which (48°C) P-188 fails to protect against thermal damage. These results correlate with those of Padanilam *et al.* [16], who showed that the ability of P-188 to arrest fluorescent marker molecule leakage from skeletal muscle cells is retarded at temperatures $>$45°C.

The inability of P-188 to improve the contractile ability of cells subjected to heat shock at temperatures $>$48°C may be attributed either to the direct damage to surfactants by cell-generated free radicals or to the degree of severity of the heat shock-induced cell damage. Because the phenomenon of heat shock involves a cascade of events affecting the whole cell and not only the plasma membrane, it is most likely that there is an increased degree of damage at 48°C that may involve multiple cytoplasmic and membrane components, and repair at the plasma membrane level by P-188 is not sufficient to facilitate cell recovery. Furthermore, our P-188 preconditioning experiments indicated that the denaturation of P-188 was not a plausible explanation for the ineffectiveness of P-188 at 48°C. Since, in the less severe forms of thermal and/or electrical injury, the cells are exposed to temperatures in the range of 43 to 45°C, the results of this study may have direct clinical significance.

From the morphological study presented in this paper, we show that the actin network is stabilized by treatment with P-188 and the number of cells showing normal cytoskeletal morphology following treatment with P-188 after heat shock is higher thaen that of the untreated controls. Heat shock-induced damage to the cytoskeleton can occur either through denaturation of the microfilamentous and microtubular proteins or through changes in the ionic transport across the plasma membrane [8, 13]. The two most probable explanations for the protective effect of P-188 on the cytoskeletal network of heat shocked cells are as follows. The restoration of membrane integrity by P-188 may indirectly stabilize the cytoskeletal network by providing the mechanical support required for anchoring the microfilamentous network. Indeed, Klotz *et al.* [37] have suggested that P-188 adsorbs to artificial membrane surfaces and forms a structure similar to the cytoskeleton of biological membranes, and the irreversible breakdown of the membrane is slowed. Alternatively, the pore sealing action of P-188 may prevent changes in the intracellular Ca^2 concentration, thus avoiding the activation of metabolites that can potentially damage cytoskeletal proteins. Although the exact mode of action is yet unclear, it is plausible that P-188 may involve a direct surfactant membrane interaction.

Although, the ability of P-188 to bind artificial and biological bilayer membranes has been suggested in numerous studies (see [18] for a brief review), the exact molecular mechanisms of these effects are still unclear. The most widely published hypothesis involves the hydrophobic interactions of the surfactant with the bilayer lipid membrane. It is speculated that the hydrophobic core of P-188 intercalates among the hydrocarbon domains of the bilayer lipid membrane, while the flanking hydrophilic tails remain immersed in the surrounding aqueous environment. The results of this study also suggest that P-188 may be acting acts as a membrane sealant. Specifically, since P-188 is effective in enhancing the contractile ability of cells for a certain range of heat stress it is plausible that damage to the plasma membrane promotes P-188 membrane interactions, thereby assisting cell repair and enhancing cell recovery. However, outside this threshold, at higher temperatures, heat shock-induced injury is most likely too severe with damage to cellular components other than the plasma membrane. In this case, the membrane stabilizing effect of P-188 is inadequate to affect repair of other damaged cellular components.

Another possible mechanism for the protective effect of the surfactant is that P-188 arrests the consumption of high energy catalytic compounds such as ATP. Ionic

permeabilization of the cell membranes leads to loss of cell viability. Because the majority of cellular ATP utilization is directed toward maintaining transmembrane ionic concentration differences, permeabilization would conceptually demand proportionate increases in ATP generation and utilization to maintain cytoplasmic ionic homeostasis. It is probable that in the absence of P-188, membrane permeabilization results in the rapid exhaustion of adenosine phosphates by membrane pumps which may result in catalysis of reactive oxygen species [38]. These reactive oxygen species then causes further membrane degradation through lipid peroxidation [39]. This process can be expected to lead to progressive membrane degradation, metabolic arrest, and finally, loss of cell viability [40]. Our results indicate that a P-188 membrane interaction may be involved in enhancing cell function following heat shock. It is clear that future studies investigating the mechanisms of P-188-membrane interaction are required at this time.

In summary, our results demonstrate the effectiveness of P-188 in increasing both the morphological integrity and the contractile function in cells following heat shock. There exists a degree of heat shock-induced thermal stress (45°C for 20–60 min) for which treatment with low concentrations of P-188 ($\leq$0.5 mg/ml) are effective in minimizing cell injury. Since we have applied P-188 after heat shock and used FPCLs which model contraction that occurs in skin during wound healing, these results may be potentially relevant to clinical application. In light of the fact that the cell membrane is the primary target in most physiochemical injuries, treatment with P-188 will be valuable not only for heat shock induced damage but for other membrane-related injuries as well. These results are very promising and make P-188 a potential candidate for clinical protocols in electrical and/or burn trauma treatment. It should be noted that the time of application of P-188 following injury may be critical for clinical application, studies are currently in progress to investigate the efficacy of P-188 following different durations of application after heat shock. Future studies investigating the mechanisms of P-188-membrane interaction are required at this time. This information would aid in the development of better therapeutic treatment schemes for electrical trauma and burn victims, and in the design of more effective copolymer surfactants.

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