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Glucocorticoids suppress fibroblast apoptosis in an *in vitro* thermal injury model

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ABSTRACT

The wounds of full- and deep partial-thickness burns result in hypertrophic scars and lead to skin contracture more severely than those of superficial partial-thickness burns. Therefore, preventing burn progression may help improve the aesthetic and functional outcomes after healing. Although a number of studies have focused on elucidating the underlying mechanisms of and preventing burn wound progression, it is still difficult to rescue burned dermis unless early tangential excision is performed. To investigate the underlying mechanisms of and prevent cell death of heat-injured fibroblasts, we developed an *in vitro* experimental model of heat-injured fibroblasts. We confirmed that heating at 55°C for 30s caused fibroblast necrosis immediately after heating, whereas heating at 46°C for 30s induced apoptosis 24h after heating. We also found that the supplementation of 100ng/ml betamethasone to the culture medium after heating decreased the number of apoptotic cells and increased that of live cells. Our studies suggest that glucocorticoids suppress apoptosis of heat-injured fibroblasts and may be useful for preventing burn wound progression.

1. Introduction

In a cutaneous burn wound, there are three zones of thermal injury: the central zone of coagulation, the intermediate zone of stasis, and the outer zone of hyperemia [1]. Because the blood flow in the intermediate zone of stasis decreases naturally after thermal injury, removing the central zone of coagulation and performing immediate skin grafting is one way to rescue the zone of stasis [2]. Without treatment, the tissues in the zone of stasis will gradually die. This change is known as burn wound progression or conversion [3]. It is believed that the loss of blood flow is the main cause of tissue destruction, including cell death of fibroblasts [3]. Several pathologic processes have been implicated in burn progression: prolonged inflammation, microthrombosis, and hypoperfusion [3,4]. As many factors contribute to burn wound progression, the mechanisms underlying the cell death of fibroblasts have not been fully elucidated.

The cell death of fibroblasts in a burn wound is attributed to one of two distinct mechanisms: necrosis and apoptosis [5,6]. Necrosis is an irreversible process, whereas apoptosis is a programmed process and can be suppressed by inhibiting the

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apoptotic pathway [7]. It has been reported that apoptosis in this case is caused by mild hyperthermia in various types of cells including fibroblasts [8–10]. Therefore, inhibiting the apoptotic pathway activated by thermal injury might help suppress burn wound progression.

We and others have developed animal models of burn injury to study the mechanisms of burn wound progression [11–13]. To investigate the process of cell death in greater detail, we attempted to develop an *in vitro* burn model. Researchers commonly create an *in vitro* thermal injury model by heating the culture medium [8–10,14]. In these models, the heating time is approximately 10 min. A cutaneous burn usually gives rise to several seconds of heating followed by immediate cooling. Therefore, an experimental model involving the direct heating of cells might be suitable to investigate the effect of cutaneous burns on heated cells.

As topical steroids are used clinically for treating inflammatory skin diseases such as eczema and atopic dermatitis [15,16], topical steroids are sometimes also used to treat burn injuries to reduce inflammation. Indeed, the efficiency of glucocorticoid ointment for superficial partial-thickness burns has been reported [17,18]. In addition, glucocorticoids have been reported to be effective in preventing dermal ischemia in an *in vivo* burn model [19]. However, the mechanism underlying the rescue of cell death of fibroblasts after heat injury by glucocorticoids has yet to be clarified.

In this study, we developed an *in vitro* model of direct heat injury on fibroblasts and investigated whether or not glucocorticoids suppress fibroblast apoptosis after heat injury.

2. Materials and methods

2.1. Cell culture

Fibroblasts were harvested from rat skin dermis and cultured in Dulbecco's Modified Eagle's Medium (DMEM; SIGMA, UK)

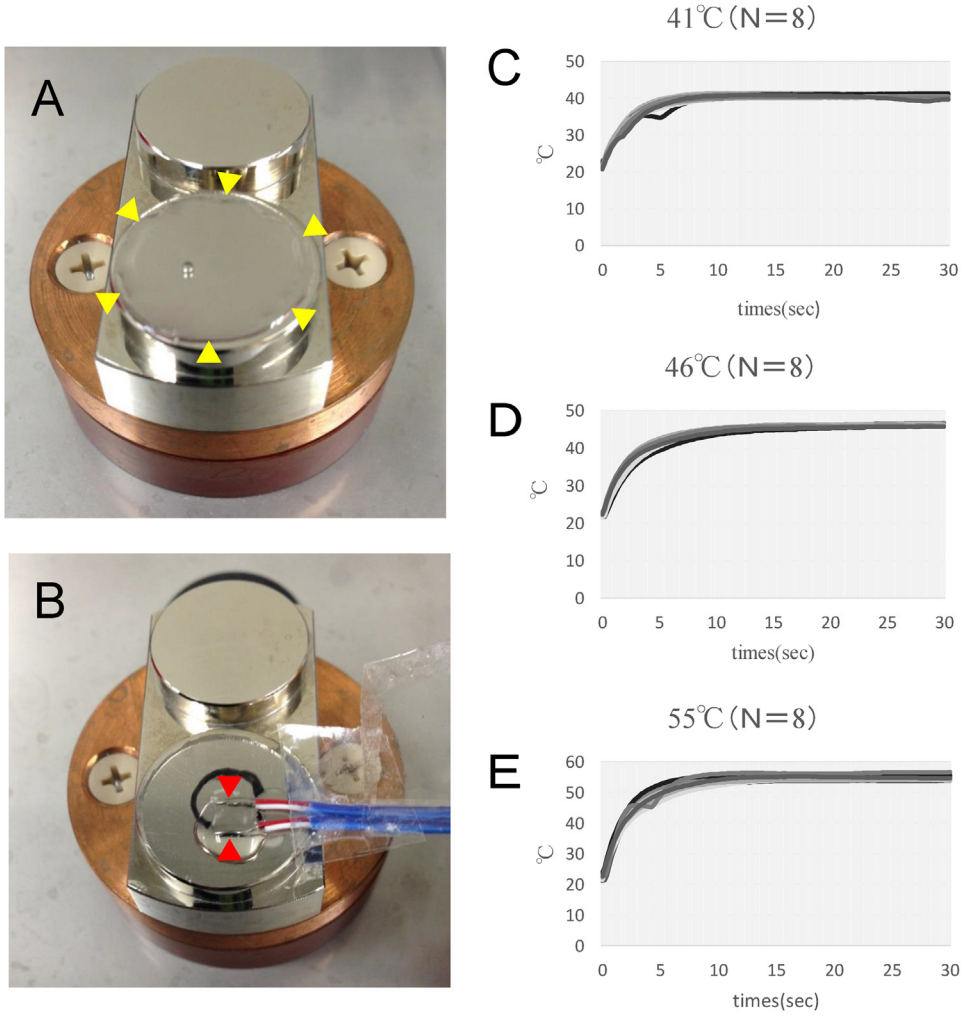

Fig. 1 – Device and settings for the heating of cultured fibroblasts. (A) The fibroblast-seeded cover slip (yellow arrowheads, 18 mm in diameter) was placed on the heated metal. (B) The temperature of the cover slip was validated using a temperature sensor (red arrowheads). (C) Temperature plot for a setting temperature of 41 °C. (D) Temperature plot for a setting temperature of 46 °C. (E) Temperature plot for a setting temperature of 55 °C.

supplemented with 10% fetal bovine serum (FBS; HyClone, USA) and 100U/ml penicillin and 100μg/ml streptomycin (Gibco, USA). As cells achieved confluence, they were subcultured. The cells were used at passage 4 or 5 in the experiment.

2.2. Experimental setup

Fibroblasts (1×10^5) were seeded on coverslips (18mm in diameter) in 12-well plates. Three days after seeding, the cells were placed on a heated metal with 50μl of phosphate-buffered saline (PBS; Gibco) for 30s (Fig. 1A). After heating, the coverslips were placed in the wells of 12-well plates with fresh DMEM supplemented with 2% FBS; the plates were incubated for 24h. The temperature of the coverslip was recorded using DETA ROGA® (Keyence, Japan; Fig. 1B).

2.3. Assessment of cell death and apoptosis after thermal injury

Twenty-four hours after thermal injury, cell death was assessed using LIVE/DEAD® Viability/Cytotoxicity Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instruction. Apoptosis and necrosis of fibroblasts were assessed using the Apoptotic/Necrotic/Healthy Cells Detection Kit (PromoCell, Germany) according to the manufacturer's instruction. The stained cells were observed under a Biorevo BZ-9000 microscope (Keyence), and the number of fluorescence-positive cells was counted using the MicroCell Count software program (Keyence).

2.4. Application of betamethasone

Betamethasone (100ng/ml, Shionogi, Japan) was added to fresh culture medium immediately after thermal injury.

2.5. Statistical analyses

The numbers of live, necrotic, and apoptotic cells at each temperature were analyzed by using the Steel-Dwass' test. The numbers of live, necrotic, and apoptotic cells between the groups with or without betamethasone were analyzed by using the Student *t*-test. A P-value of 0.05 was considered as statistically significant.

Table 1 – Evaluation of setting temperature.

Temperature (°C)	Number	Mean	SD	Range
41	8	40.96	0.42	40.35–41.50
46	8	46.29	0.31	45.80–46.70
55	8	54.91	0.84	54.30–56.65

3. Results

3.1. In vitro thermal injury model for fibroblasts

To establish an *in vitro* thermal injury model for cultured cells, we used a 20 mm-diameter metal sheet whose temperature could be controlled digitally (Fig. 1A). The actual temperature of the coverslip on the heated metal was validated using a temperature sensor (Fig. 1B). The setting temperature was calculated for the temperatures 41°C, 46°C, and 55°C at eight different times for each one (Fig. 1C–E). Table 1 shows the results of maximum values for the setting temperature. We consider that the temperature is stable and the range error is small; therefore, this *in vitro* burn model has reproducibility.

To determine the appropriate temperature for evaluating cell death by thermal injury, we first attempted to determine the duration of heating. Heating the cells for more than 30s at 60°C resulted in drying of the cells. Therefore, we set the duration of heating as 30s to evaluate cell death due to thermal injury at different temperatures. We then performed the experiments at temperatures from 40 to 60°C to evaluate cell death due to thermal injury. By heating the cells at 41°C, only a few dead cells were detected at 24h after heating (Fig. 2A). By contrast, by heating the cells at 55°C, only a few live cells were detected at 24h after heating (Fig. 2C). By heating the cells at 46°C, the numbers of live and dead cells were between the respective numbers obtained for heating at 41°C and 55°C (Fig. 2B). These results suggest that the amount of cell death depends on the heating temperature.

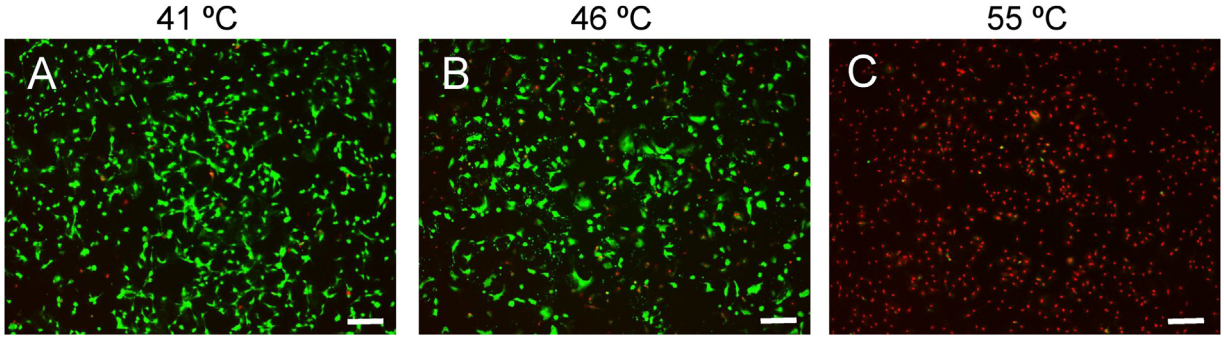

Fig. 2 – Optimization of temperature for inducing necrosis of fibroblasts. (A–C) Representative images of staining of the live (green) and dead (red) cells at 24h after heating. A few dead cells were detected at 41°C (A). The number of live cells at 46°C (B) was decreased compared to that at 41°C. A few live cells were detected at 55°C (C). Scale bars, 300μm.

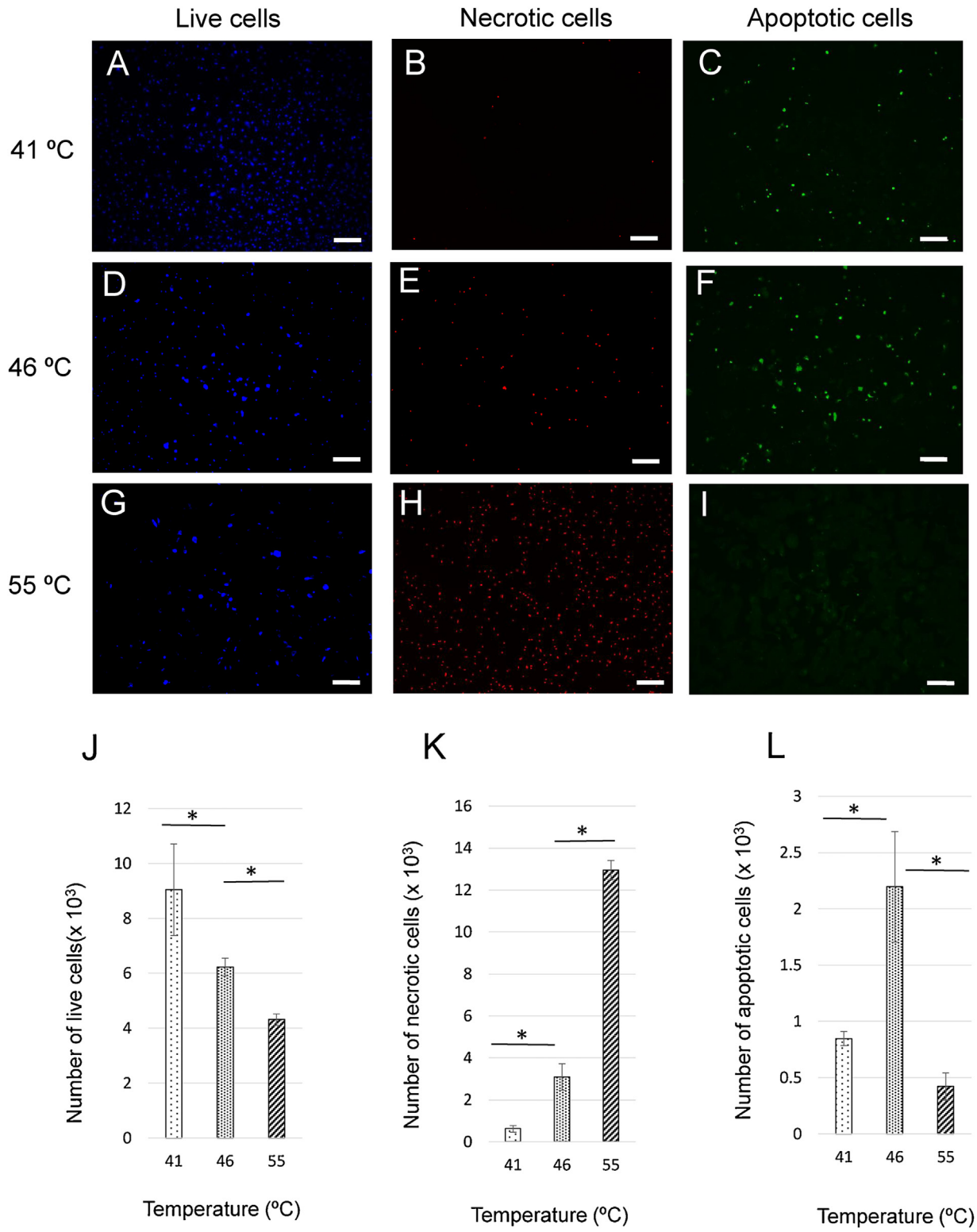

Fig. 3 – Detection of apoptotic fibroblasts caused by heating. (A-I) Representative images of staining of the live (blue), necrotic (red), and apoptotic (green) cells at 24h after heating. A few necrotic cells were detected at 41°C (B), and apoptotic cells were also detected (C). Both necrotic and apoptotic cells were detected at 46°C (E and F). A few apoptotic cells were detected at 55°C (I). (J-L) Quantification of the numbers of live cells (J), necrotic cells (K), and apoptotic cells (L). N=3, *: $p<0.05$. Scale bars, 300 μm.

3.2. *Apoptosis of fibroblasts by thermal injury*

To investigate the type of cell death induced by thermal injury, we performed staining with Ethidium Homodimer III to detect necrotic cells and with Annexin V to detect apoptotic cells. When the cells were heated at 41°C, a few apoptotic cells were detected (Fig. 3C). By heating the cells at 46°C, the numbers of both necrotic and apoptotic cells were increased compared to those heated at 41°C (Fig. 3B and C). By heating the cells at 55°C, the number of necrotic cells was markedly increased, whereas few apoptotic cells were observed (Fig. 3H and I). These results suggest that apoptosis of fibroblasts by thermal injury was caused by heating at lower temperatures than the temperatures that cause necrosis.

3.3. *The effect of betamethasone on the viability of fibroblasts after thermal injury*

Given that topical steroids are known to be effective in treating burn injury [17,19], we hypothesized that glucocorticoids might suppress apoptosis caused by thermal injury. When we added 100 ng/ml of betamethasone to the culture medium immediately after heating the cells at 46°C, the number of live cells at 24 h after heating was significantly increased (Fig. 4A). The number of necrotic cells was not changed between the two groups, but the addition of betamethasone reduced the number of apoptotic cells compared with no supplementation (Fig. 4B and C). These results suggest that glucocorticoids suppress the apoptosis of fibroblasts induced by thermal injury.

4. Discussion

In this study, we developed an *in vitro* thermal injury model of fibroblasts to analyze the cell death after heating. We used a temperature-controlled metal device and placed the cover slip on the heated metal to produce a direct burn model. After placing a culture plate with medium on the heating device, the temperature of the medium increases gradually, taking about 10 min to induce thermal injury of the cells [14]. In another *in vitro* burn model, a glass-bottom culture dish without medium is placed on a base of glass preheated using a microwave oven for 30 s [20]. Because the temperature of the metal device we used in this study is controlled digitally and kept constant before the experiment, it was easy to reproduce the thermal injury model of cultured cells.

Deep partial-thickness burn wounds tend to progress to full-thickness burn wounds unless treated. This is called secondary burn wound progression or conversion [21]. As deep partial-thickness burns change to full-thickness burns within several days after the burn, immediate surgery such as excision of the necrotic tissue and skin grafting can rescue the tissue that would otherwise be destroyed by burn progression [22,23]. The wounds of full- and deep partial-thickness burns result in hypertrophic scars and lead to more severe skin contracture than superficial partial-thickness burns. Therefore, preventing burn progression can improve the aesthetic and functional outcomes after healing. Many factors that may provoke burn progression have been reported, including edema, inflammation, free radical damage, vasoconstriction, and hypoperfusion

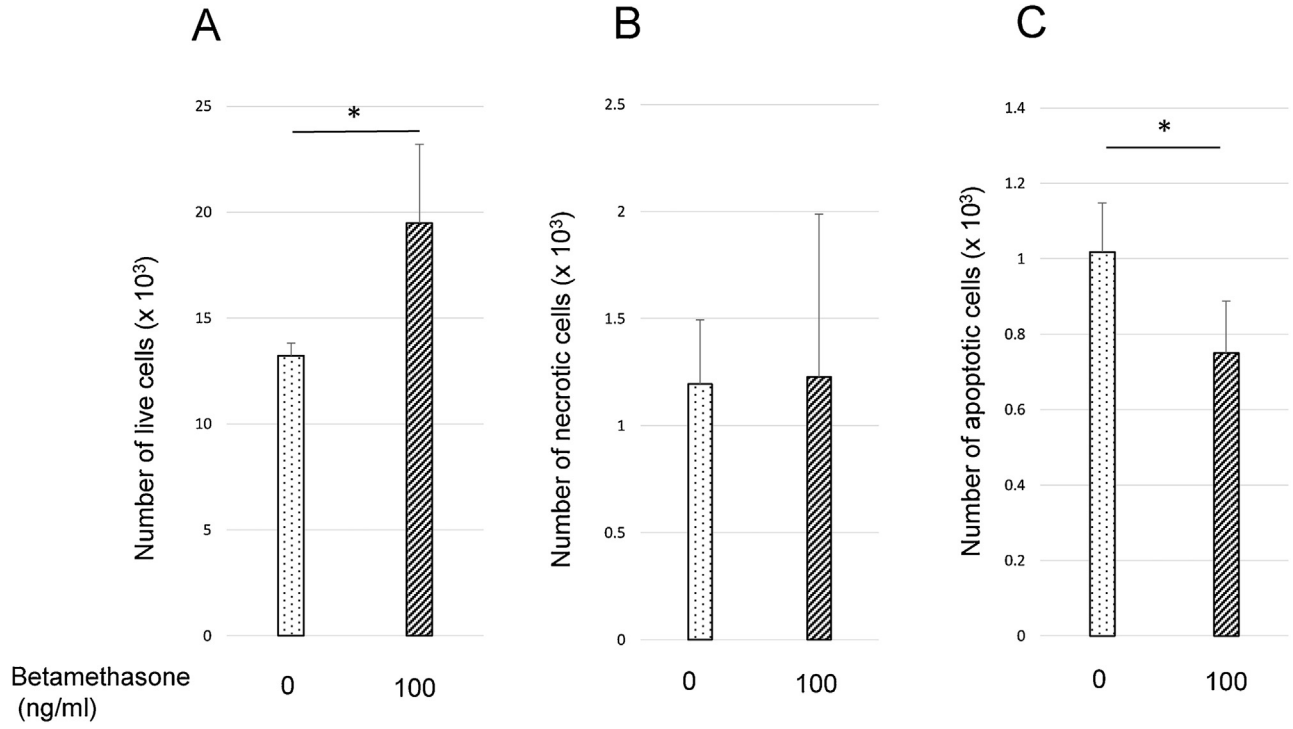

Fig. 4 – The effect of betamethasone on fibroblasts heated at 46°C. (A–C) Quantification of the numbers of live cells (A), necrotic cells (B), and apoptotic cells (C) at 24 h after heating. N=3, *: $p < 0.05$.

[3]. Regardless of the mechanism, the tissues in the zone of stasis are not destroyed immediately after the burn injury; therefore, a number of studies have focused on investigating the process of cell death in the dermal tissue, including fibroblasts, during burn progression [20].

Several reports have described the existence of both necrotic and apoptotic cells in the ischemic zone of deep partial-thickness and full-thickness burns [5,12]. These papers suggest that the cell death that occurs during burn progression is caused by a loss of blood flow. However, dermal and epidermal tissues do not die when deprived of blood flow for only a couple of days: for instance, full-thickness skin grafts can survive for 2–3 days until reperfusion occurs and engraft without tissue destruction [24]. Therefore, initial burn progression might be attributed to cell death not by ischemia but by direct thermal injury. Indeed, apoptotic cells were detected 24 h after direct thermal injury in our *in vitro* experiments (Fig. 3). These results indicate that the apoptosis of fibroblasts by direct thermal injury is involved in burn wound progression.

The clinical use of topical steroids to treat burn injuries was first described in a letter [17]. Experimentally, topical steroid inhibited the inflammation and improved the dermal perfusion after a burn [25]. In addition, it has been reported that glucocorticoids protect against TNF-α-induced apoptosis in mouse fibroblast L-929 cells [26,27]. Therefore, we speculated whether or not glucocorticoids could suppress fibroblast apoptosis induced by thermal injury. We tested the effects of a glucocorticoid on heat-injured fibroblasts at concentrations of 1, 10, 100, and 1000 ng/ml of betamethasone, and 100 ng/ml of betamethasone showed an anti-apoptotic effect (Fig. 4). These results demonstrated that glucocorticoids reduce the apoptosis of heat-injured fibroblasts *in vitro*. Although further studies are needed to evaluate the effects of glucocorticoids on the apoptosis of heat-injured fibroblasts *in vivo*, glucocorticoids might alleviate burn wound progression by promoting the survival of injured fibroblasts. Although there is an article on an agent having a similar effect, it is described that astaxanthin protects early burn wound and inflammation and apoptosis in rat models. Astaxanthin is a natural carotenoid widely distributed in marine organisms such as algae, crustaceans, salmon, shrimp, and crabs [28]. This agent seemed to be useful for the early treatment of burns.

There is the problem of side effect when using glucocorticoids; this results in infection. However, we think that the advantage outperforms the disadvantage for the short-term use of glucocorticoids. There is a report on the use of a kind of glucocorticoid for superficial partial-thickness burns. Hossain described that 5 of 49 patients had infection after 8–12 days of topical treatment [18]. We speculate whether short-term use would decrease infection. The purpose of using it is to suppress initial apoptosis of fibroblasts and initial wound burn progression.

5. Conclusion

We developed an *in vitro* thermal injury model of fibroblasts. Using this model, we showed that glucocorticoids suppress fibroblast apoptosis induced by thermal injury. The use of steroids to treat burn injuries might prevent burn wound progression by suppressing fibroblast apoptosis.

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