Original Article

Extracorporeal high-pressure therapy (EHPT) for malignant melanoma consisting of simultaneous tumor eradication and autologous dermal substitute preparation☆

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A R T I C L E   I N F O

A B S T R A C T

Surgical resection of skin tumors leads to large defects in surrounding normal tissues, which should be reconstructed thereafter using the patient's own tissues taken from the other site. Our challenge is to solve this problem in dermal malignant melanoma (MM) by a novel process, named extracorporeal high pressure therapy (EHPT), in which the tissue containing tumor is resected and pressurized, and the treated tissue is re-transplant back to the same position as a tumor-free autologous dermal substitute. The key points are complete tumor death and preservation of native extra cellular matrix (ECM) by the hydrostatic pressure. We found that high hydrostatic pressure at 200 MPa for 10 min at room temperature is completely cytocidal against MM cells in suspension form, in monolayer form, and even in the solid tumor form. MM tumor-bearing nude mice were established by injected human MM cells intradermally and treated by EHTP. The denaturation of the dermal extra cellular matrices was so mild that the pressurized skin was well engrafted as tumor free autologous dermal tissues, resulting in the complete eradication of the MM without any unnecessary skin reconstruction surgery. This very simple and short pressing treatment was proved to make the tumor tissue to the transplantable and tumor-free autologous dermal substitute, which can be applicable to the other temporally resectable tissues.
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1. Introduction

Our challenge is to develop a novel therapeutic modality for malignant melanoma (MM) treatment. MM is a severe, high-grade malignancy that is associated with an extremely poor prognosis. The recent immunotherapy for MM costs a lot, needs longer treatment time, and is not a radical treatment yet. The goal of their treating is to obtain safe and complete cure combined with satisfactory reconstruction of the tissue using autologous tissue-derived scaffolds. The surgical resection is the most general and reliable treatment for malignant tumors. However, large tissue defects sometimes remain after the surgical resection because malignant tumors should be eradicated over a wide area including the surrounding normal tissues to achieve curative treatment. These defects should be reconstructed somehow. In the case of cutaneous,

subcutaneous and/or osseous tissue tumors, reconstructions using the patient's own tissues as scaffolds are conducted to restore the function or appearance. The sacrifice of the normal tissues thus remains a problem associated with reconstructive surgery. Here, we proposed a reconstruction strategy that is a re-implantation of the excised tissue as autologous tissue-derived scaffolds to the same position after inactivating the cancer cells. The key challenges are how to completely kill the tumor cells and how to simultaneously preserve the native extracellular matrix.

In the case of the osseous tumors, it is possible to re-implant resected bones back into patients after the inactivation of tumor cells by heating (autoclaving), pasteurizing, or irradiation [1–4]. However, since these treatments induce an unacceptable degree of denaturation of the extracellular matrix proteins as well as the tumor death, they cannot be applied to the soft tissues [5]. We recently proposed a safe and mild tissue treatment process using high hydrostatic pressure (HHP) [6–9]. HHP treatment at 600 MPa and higher has been reported to inactivate (to kill) cells, fungi, viruses, and pathogens [10]. We have been reporting the transplantation results of the decellularized tissue treated at 1000 MPa and thoroughly washed [11–16]. More recently, we found an optimized HHP condition of 200 MPa for 10 min that completely inactivated mammalian cells without damaging the matrices of porcine skin, normal skin, or human nevus tissue [17–21]. According to these findings, we came up with a new extracorporeal high pressure therapy (EHPT) for MM that combines cancer treatment and reconstructive surgery, in which the MM tissue is excised, inactivated at 200 MPa, and re-implanted at the original site. In the present study, the cytolytic effect of HHP to the MM cells was confirmed *in vitro* and *in vivo*, and its therapeutic efficacy for melanoma-bearing mouse was proved.

2. Materials and methods

2.1. The preparation of MM cells

Melanoma cell lines derived from human malignant melanomas (Public Health England Culture Collection, UK) were utilized for these studies. For the primary culture of malignant melanoma cells, DMEM supplemented with 15% fetal bovine serum (FBS, Life Technologies Japan, Ltd., Tokyo, Japan) with 100 units/mL penicillin, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B (Life Technologies Japan, Ltd., Tokyo, Japan) was added and incubated at 37 °C, 95% humidity, and 5% carbon dioxide. The medium was changed every two or three days. After one week, the MM cells were dissociated using TripLE Express (Life Technologies Japan, Ltd., Tokyo, Japan) and passaged. The MM cells were suspended in CELLBANKER 1 (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) and cryopreserved after the second or third passage.

2.2. The HHP treatment of MM cells

The cryopreserved MM cells were rapidly thawed in a water bath, and 1×10^6 cancer cells were seeded and cultured on a 10 cm culture dish with DMEM supplemented with 15% FBS and antibiotics. The medium was changed every two or three days until confluence. After dissociation, a suspension of 10^5 cells/mL in the culture medium was prepared. The suspension of cancer cells was divided equally into three parts, which were each packed in a plastic bag with the cell culture medium. One bag was preserved at room temperature without pressurization, another bag was pressurized at 100 MPa, while the remaining bag was pressurized at 200 MPa. The bags in the pressurization groups were placed in the inner cavity of the pressure-tight cell and the pressure was increased to 100 or 200 MPa over a period of a few minutes and maintained at the target pressure for 10 min, then decreased to atmospheric pressure over a period of a few seconds. One hundred μL of the cell suspension from each group (n = 3 in each group) and 1 mL of the culture medium was seeded into a 24-well cell culture plate (AGC Techno Glass Co.,Ltd., Tokyo, Japan) and cultured for 24 h. The cellular morphology and attachment were then observed under a microscope (Nikon TE-200; Tokyo, Japan) after 3 and 24 h.

2.3. The Live/Dead staining of MM cells

A Live/Dead staining solution (Live/Dead Cell Staining Kit II; PromoCell GmbH, Germany) of 2 μM Calcein-AM/4 μM EthD-III (Ethidium homodimer III) was prepared by adding 5 μL of 4 mM Calcein-AM and 20 μL of 2 mM EthD-III to 10 mL of PBS in accordance with the manufacturer's instructions. One mL of the cell suspension of each group was centrifuged at $400 \times g$ for 5 min and the collected cells (1×10^5 cells) were washed with PBS. The suspension was centrifuged again, and the cells were suspended in 1 mL of prepared Live/Dead solution. The cells were incubated at 37 °C for 1 h, and 100 μL of each suspension was put on a glass slide. Images were then obtained using an Olympus FluoView confocal laser scanning microscope (Olympus Corp., Tokyo, Japan). We seeded 1×10^5 cancer cells without pressurization on the chamber of a Chamber Slide II (AGC Techno Glass Co., Ltd., Tokyo, Japan) and cultured them until confluence. The media was discarded and a Live/Dead staining solution was added to the chamber and images were obtained after incubation for 1 h. Another chamber was then packed in a plastic bag and pressurized at 200 MPa. This chamber was also incubated with the solution for 1 h and observed.

2.4. The viability of HHP-treated MM cells

We quantitatively evaluated the viability of cells without pressurization and after pressurization at 100 MPa and 200 MPa using a WST-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-[4-nitrophenyl]-2H-5-tetrazolio]-1,3-benzene disulfonate sodium salt) assay. One hundred microliters of the remaining cell suspension of each group was added to each well (n = 7 in each group of cancer cells) of 96-well plates (AGC Techno Glass Co., Ltd., Tokyo, Japan). The plates were then incubated at 37 °C for 15 min. After incubation, 10 μL of WST-8 assay reagent (Cell Count Reagent SF, Nacalai Tesque, Inc., Kyoto, Japan) was added to each well and incubated at 37 °C for 1 h. The plate was then gently shaken, and the absorbance of the medium was read using a microplate reader (model 680; Bio-Rad Laboratories, Inc., Hercules, CA) at a test wavelength of 450 nm and a reference wavelength of 650 nm. The absorbance of each medium in the vacant wells (n = 7) was also measured and this absorbance was used as an arbitrary zero point.

2.5. The growth of the HHP-treated MM tumors

A suspension of 10^6 cells/mL cancer cells was prepared in the culture medium. One hundred microliters of cancer cells were intradermally injected into both sides of the back of 6-week-old male BALB/c nude mice (Charles River Laboratories International, Inc., Takatsuki, Japan) (n = 5). During the injection, the mice were anesthetized by the inhalation of 2% isoflurane (Wako Pure Chemical Industries, Ltd., Osaka, Japan). The mice were sacrificed by carbon dioxide inhalation and specimens were taken at 12 weeks after implantation. The grown MM tumors (mentioned above) were divided into smaller parts. Half of them were preserved at room temperature without pressurization and the other half were pressurized at 200 MPa. The tumors were implanted into the subcutis on backs of Six-week-old male BALB/c nude mice (n = 10). The mice

were sacrificed by carbon dioxide inhalation and specimens were observed, taken and weighed at 7 weeks after implantation.

2.6. The Live/Dead cell staining of HHP-treated MM tumors

The grown tumors (mentioned above) were each divided into smaller parts and placed into separate bags. One bag was preserved at room temperature without pressurization; the other was pressurized at 200 MPa. The tumors were suspended into 1 mL of prepared Live/Dead solution. The tumors were incubated at 37 °C for 1 h and each specimen was put on a glass slide. Images were then obtained using an Olympus FluoView confocal laser scanning microscope (Olympus Corp., Tokyo, Japan).

2.7. The histological assessment of the HHP-treated MM tumors

The hematoxylin and eosin (HE) and S-100 immunohistochemical staining of the MM tumors was conducted just after HHP treatment and at 7 weeks after transplantation. The MM tumors were fixed with 10% neutral-buffered formalin solution and embedded in paraffin blocks. Sections of 5 μm in thickness from the central area of each sample were stained with HE. Other sections were used for the immunohistochemical staining of anti-S-100 protein. After deparaffinization and rehydration, the sections were immersed in 3% hydrogen peroxide (H_2O_2; Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 10 min to block endogenous peroxidase activity. The sections were rinsed in DW and Tris Buffered Saline (TBST; Sigma Aldrich Japan. Co. Ltd., Tokyo, Japan) with 0.05% Tween® 20 (Polyoxyethylene Sorbitan Monolaurate, Nacalai Tesque, Inc., Kyoto, Japan) and 0.15 M NaCl (Sigma Aldrich Japan. Co. Ltd., Tokyo, Japan), then protein blocking Serum-Free (Code X0909, Dako Japan Co. Ltd., Tokyo, Japan) was applied for 5 min to block non-specific protein binding. The sections were applied with rabbit policlonal anti-type S-100 antibodies (dilution 1:4000; Code Z0311, Dako Japan Co. Ltd., Tokyo, Japan) as primary antibodies for 30 min at room temperature. The sections were rinsed in TBST, then ENVISION$^+$ Single Reagents (Dako Japan Co. Ltd., Tokyo, Japan) were applied for 30 min. The sections were then rinsed with TBST, exposed to DAB (3-3′-diaminobenzidine tetrahydrochloride; Nichirei Bioscience Inc., Tokyo, Japan) and counterstained with hematoxylin.

Microphotographs were taken using a fluorescent microscope (Biorevo BZ-9000; Keyence, Co., Osaka, Japan) at 200× magnification.

2.8. Extracorporeal high-pressure therapy (EHPT) for mice MM model

An MM cell suspension (10^6 cells/mL) was prepared in the culture medium. One hundred microliters of cell suspension was intradermally injected into the backs of six-week-old male BALB/c nude mice (Charles River Laboratories International, Inc., Takatsuki, Japan) (n = 10). During the injection, the mice were anesthetized by the inhalation of 2% isoflurane (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Twenty-four hours later, the mice (n = 5) were anesthetized by the inhalation of 2% isoflurane and the region of back skin within 3 mm of the injection site was excised. The five specimens were then packed in a plastic bag filled with normal saline solution (NSS; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) and pressurized at 200 MPa using the above-mentioned procedure. The pressurized MM tumor tissues were then re-implanted into each mouse and the MM growth was observed for 12 weeks.

2.9. Statistical analysis

Statistical significance was assessed using the Tukey—Kramer multiple comparisons test. All data are expressed as the mean ± standard error (SE). P values of <0.05 were considered to indicate statistical significance.

2.10. Ethics

The HHP treatment was performed at the National Cerebral and Cardiovascular Center Research Institute and animal experiments were performed at Kyoto University. Our experimental protocol was approved by the Animal Research Committee, Kyoto University Graduate School of Medicine and the ethics committees of the National Cerebral and Cardiovascular Center. The number of animals used in this study was kept to a minimum and all possible efforts were made to reduce their suffering in compliance with the protocols established by the Animal Research Committee.

3. Results

3.1. Inactivation of cultured MM cells in suspension and monolayer by HHP

Fig. 1 shows the effect of HHP on MM cells. Live/Dead staining of the treated cell suspension showed that the untreated and 100 MPa-treated MM cells for 10 min were stained in green and that the 200 MPa-treated MM cells were stained in red 1 h after pressing. Since the Live/Dead assay is based on the elastase activity and cell membrane disruption, which may not necessarily correspond to the real cell live/death, the cells were cultured up to 24 hr. Morphology of MM cells after 3 h and 24 h is also shown in Fig. 1. The untreated and 100 MPa-treated MM cells attached to the dish and began to proliferate at 3 h, while 200 MPa-treated MM cells floated and did not attached at all, even after 24 h. Fig. 2 shows the WST assay results. Cells treated 100 MPa showed same absorbance as the control. After 200 MPa of HHP treatment, the mitochondrial enzyme activity was largely reduced but clearly remains slightly. This is a limitation of WST method that a part of the enzymatic activity remains even upon the cell death. For our purpose, the complete cell death is extremely important for compete cure of MM. In addition to the cell suspensions, the monolayer cultured MM cells on the chamber slides were also found to be greatly inactivated (Fig. 3). Most of the 200 MPa-treated MM cells were detached from the chamber slide, and the attaching cells were stained in red.

3.2. Inactivation of MM solid tumors by HHP

Inactivation of MM solid tumors was also confirmed in addition to that of MM cell suspension/monolayer. Human MM cells were intracutaneously injected into nude mice and allowed to grow into solid tumors of about 20 mm in diameter. The tumor was excised and pressurized under a given condition and stained with Live/Dead. Surprisingly, in unpressurized MM solid tumors (indicated as control), majority of cells were stained in red with small number of viable green cell. Because of the very fast growth of the MM cells, the MM solid tumor is highly necrotic but this small number of cells is sufficient for the tumor growth after inoculation to mice, resulting in the death of animals (Fig. 4, left). In contrast, after 200 MPa treatment, no cells were viable and all cells were stained in red (Fig. 4, right). This is one of the biggest merit of the HHP, and HHP can treat even the center part of the solid tumor according to Pascal's law. Untreated and pressurized MM tumors were re-implanted into the subcutis of nude mice for 7 weeks. Fig. 5
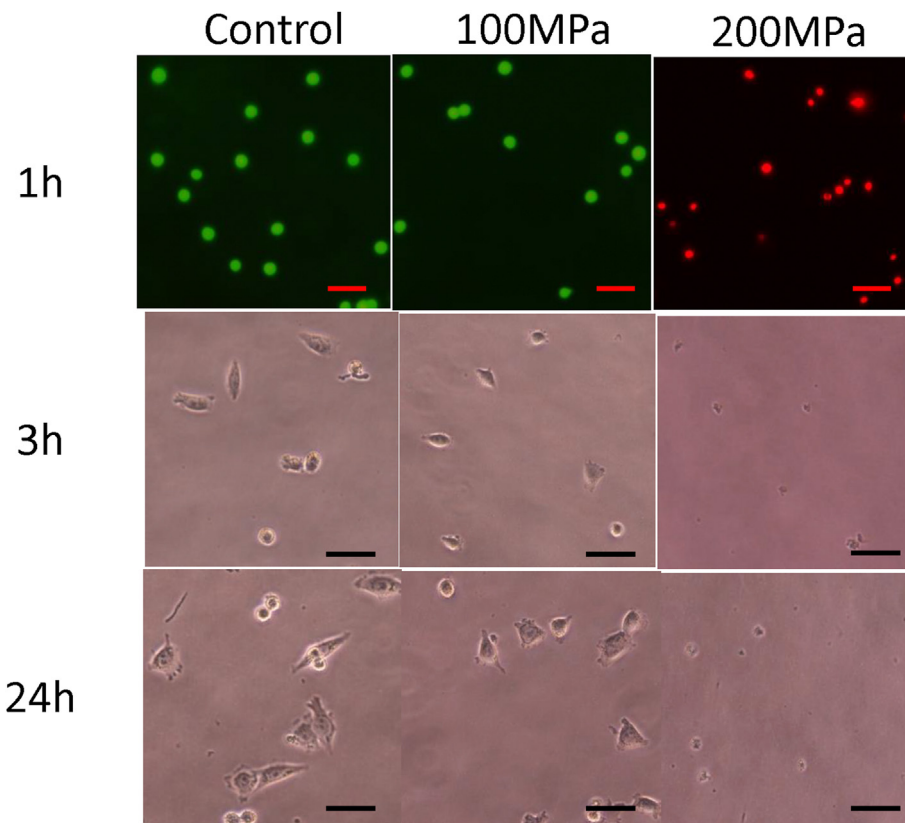
Fig. 1. The effects of HHP on MM cells in suspensions. The Live/Dead staining of MM cells at 1 h after HHP treatment, and phase-contrast microscopic observation after 3 h and 24 h of cell culture.

shows the gross appearance of the control and 200 MPa-treated tumor specimens before and after 7 weeks implantation. Unpressurized MM sold tumor clearly grew in 7 weeks. The increase in tumor weights was measured and shown in Fig. 6. The control tumors grew to approximately 10 times larger than the original size, while the 200 MPa treated tumors did not grow and almost disappeared within 7 weeks.

HE-stained sections of MM solid tumors before and after transplantation was shown in Fig. 7. Untreated MM tumors before implantation showed a distinctive large nuclear body, anisokaryosis, a polygonal shape, and sheet formation, which are the characteristic appearances of anaplastic tumor cells. Numerous mitotic cells and severe necrosis were also observed. The pressurization of MM tumors at 200 MPa resulted in a morphological change for large number of polygonal cells, however, the other

trends were the same as observed in the controls. At 7 weeks post implantation, the untreated MM tumors showed the same distinctive large nuclear bodies, anisokaryosis, and polygonal cells,
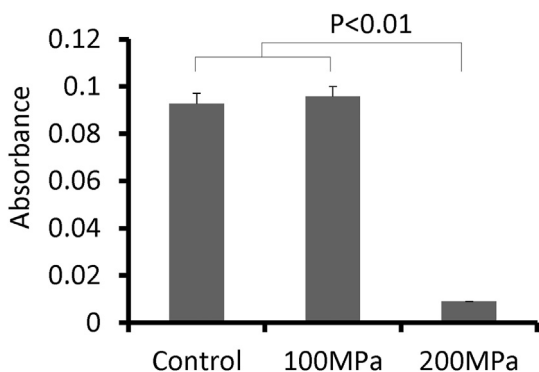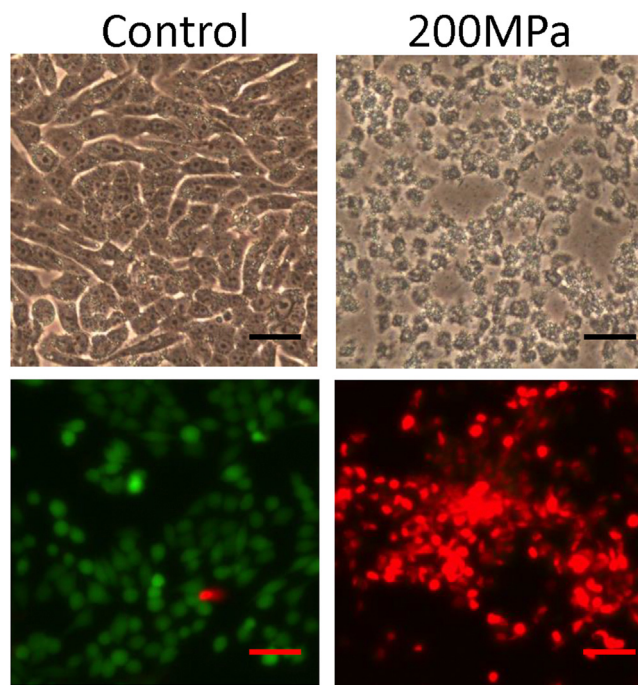

Fig. 3. Phase-contrast microscopic observation and Live/Dead staining of the monolayer cultured MM cells after 200 MPa-HHP treatment.

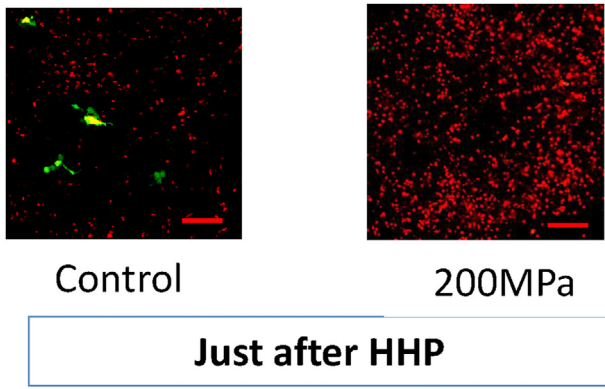**Fig. 2.** WST assay results for 100 and 200 MPa-treated MM cell suspensions.

Fig. 4. Live/Dead staining of the pressurized and control MM solid tumor tissue before implantation.

as before transplantation. In 7 weeks, the necrotic area in the untreated MM tumor increased and occupied approximately 3/4 of the total tissue, due to rapid tumor growth. In contrast the

200 MPa-treated MM tumor was completely eradicated and formed a histiocytic granuloma within 7 weeks. A calcareous deposition was also observed around the necrotic focus of the tumor (data not shown). Parts of the calcareous deposition contained the foreign-body giant cells, foreign-body granuloma, and hyaloid fibrous scar tissue. S-100 immunohistochemical staining for melanocytic cells showed that the tumor cells formed a mosaic pattern in control and 200 MPa specimens before transplantation, and at 7 weeks, a reduced number of positive cells was observed in the untreated MM tissue, while all of the melanocytic cells disappeared after HHP treatment at 200 MPa (Fig. 8).

3.3. Extracorporeal high-pressure therapy (EHPT) in the mouse model of MM

Fig. 9 shows the proof of our EHPT concept in a nude mouse MM tumor model. Human MM cells were intradermally injected into the backs of nude mice (Fig. 9A). The control groups were observed without any treatment for 12 weeks (Fig. 9F). In the 200 MPa group, the tissue within 3 mm of injection site was resected at 24 h after injection (Fig. 3B and C). The specimens were then pressurized at
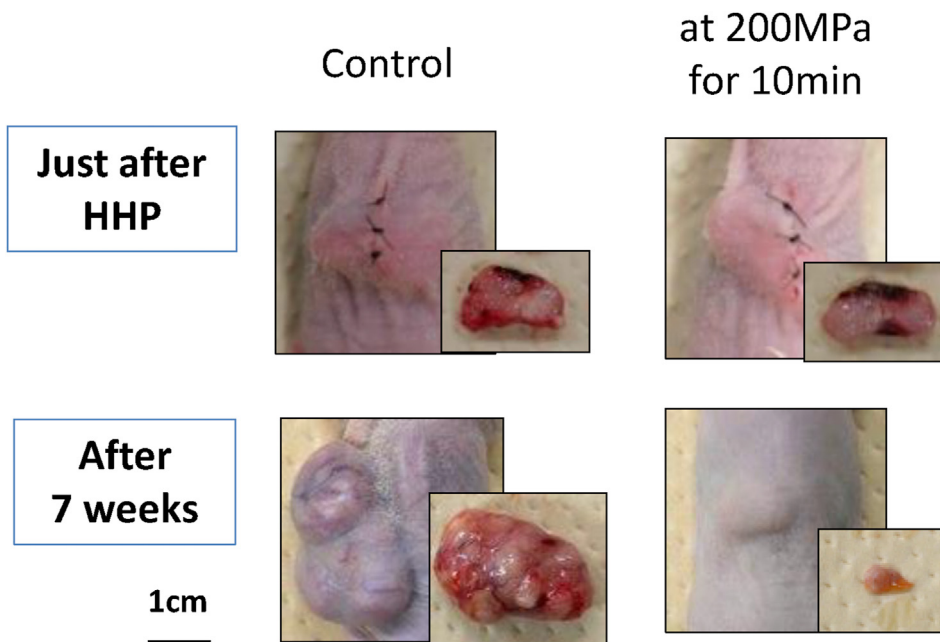

Fig. 5. The gross appearance of the untreated and HHP-treated MM solid tumor before and after 7 weeks transplantation to the nude mice.
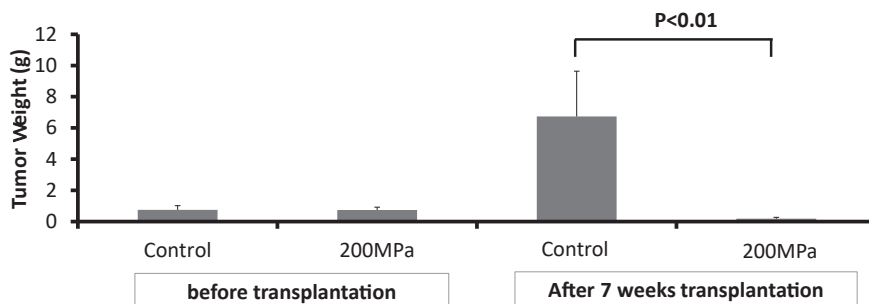

Fig. 6. Increase of the MM solid tumor in mice. A solid human MM tumor was pressurized and re-implanted into the subcutis tissue of nude mice and the tissue was weighed before and 7 weeks after transplantation.
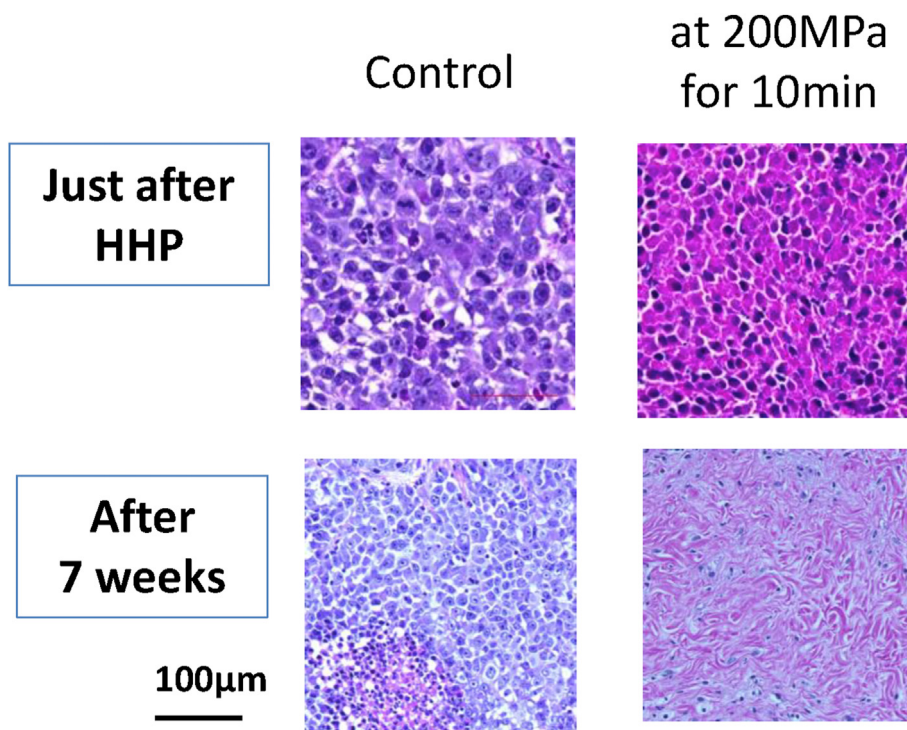
Fig. 7. HE staining of the untreated and HHP-treated MM solid tumor before and after 7 weeks transplantation to the nude mice.

200 MPa (Fig. 9D) and re-implanted (Fig. 9E). In the control group, the injected MM cells grew and formed a large crippling tumor within 12 weeks (Fig. 9F). In contrast, MM cell growth was not observed in the 200 MPa EHPT group at all, indicating the complete eradication of the MM tumors (Fig. 9G).

4. Discussion

The surgical excision of cancers, especially high-grade cancers like MM, requires a wide resection, thus resulting in both deformities and dysfunction. Dermal substitutes such as AlloDerm
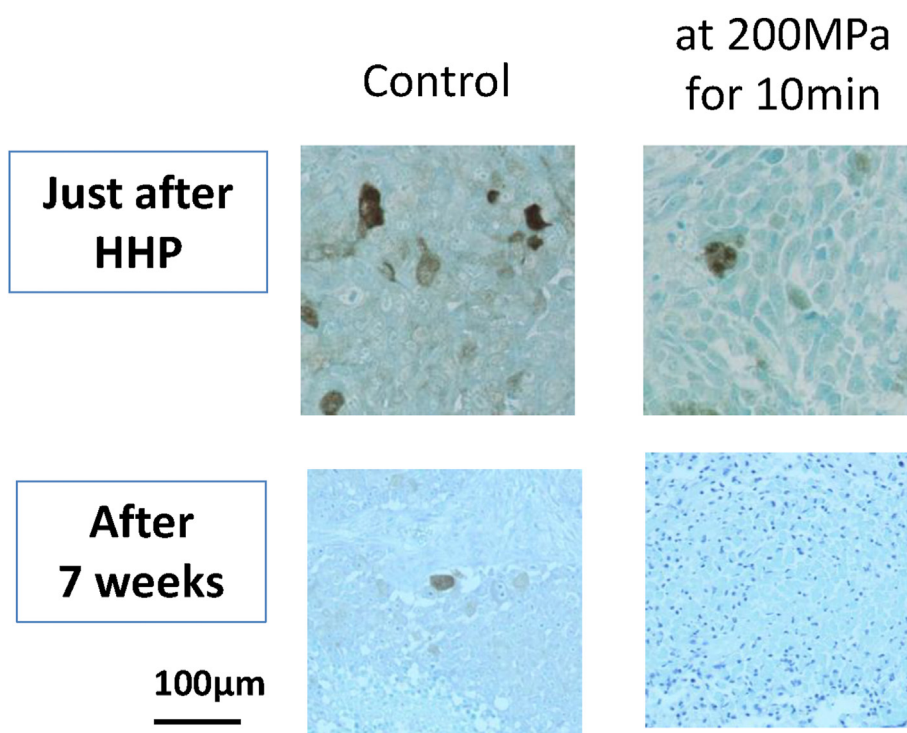

Fig. 8. S-100 immunohistochemical staining of the untreated and HHP-treated MM solid tumor before and after 7 weeks transplantation to the nude mice.
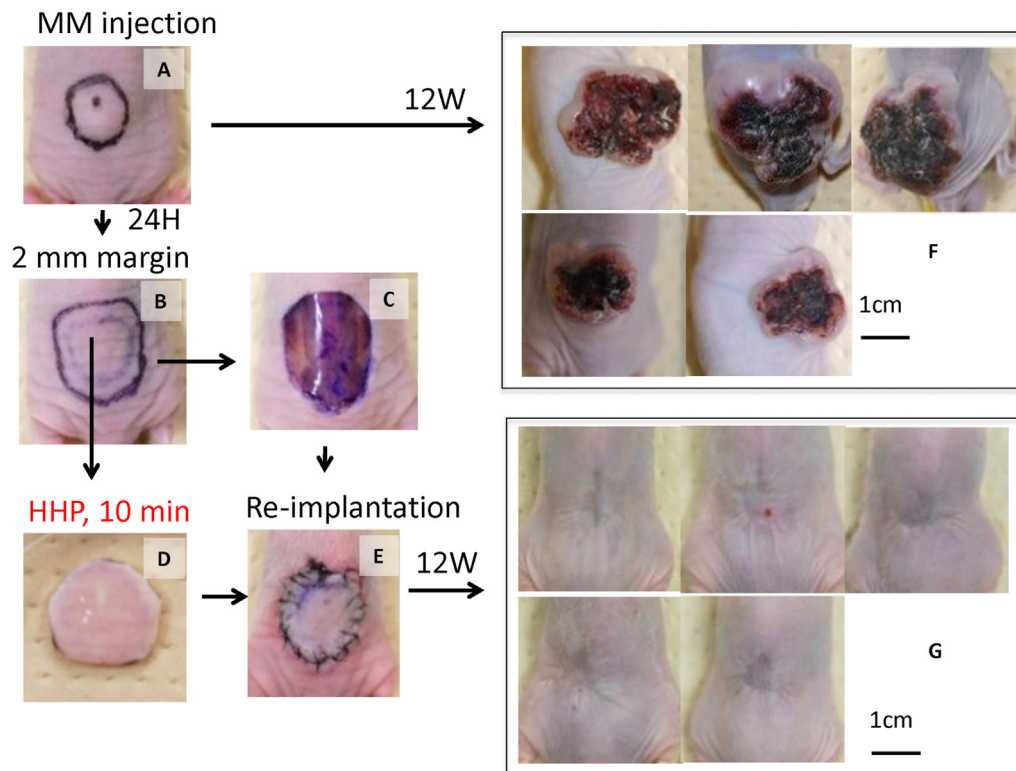
Fig. 9. The results of EHPT in the nude mouse model of MM (G). Human MM cells were intradermally injected into nude mice (A). After 24 h, the skin within 3 mm of the injection site was resected (B), pressurized at 200 MPa (D), and re-implanted (E). Control mice were (F).

(deellularized human skin, LifeCell Corp., Branchburg, N.J.), Integra (Porous sheet made of collagen, Integra Life Science Corp., Plainsboro, N.J.), and Pelnac (Porous sheet made of collagen, Gunze Ltd., Ayabe, Japan) have been used. However dermal regeneration with these dermal substitutes was not satisfactory due to their different characteristics and features from the native dermal tissue. Then, the additional surgery in combination with skin grafts after the removal of skin cancer is indispensable [22—24].

Autologous skin is the only material that can be used in the treatment of large skin defects; however, they requires unnecessary normal tissue defects. We therefore established a novel treatment to re-implant tissue as an autologous matrix after cancer eradicating treatment. This treatment was named extracorporeal high-pressure therapy (EHPT). It is theoretically possible to reconstruct any amount of large tissue defect. We have already reported the transplantation of the HHP-based decellularized tissue such as the blood vessels [13,25—28]. EHPT may be particularly useful for the renaturation of unique tissues with complicate structures, such as the vocal cords, larynx, or ovaries because there are no substitutable organs in the patients' body. The key point of this treatment is that it allows the complete eradication of cancer cells without damaging the native structure and that the treated tissue can be used as an engraftable autologous matrix.

Our results showed that tumor cells were eradicated at 200 MPa HHP for 10 min. Furthermore, we showed that the inactivated cells did not grow *in vitro* and that they did not recur *in vivo*. Furthermore, we very recently reported squamous cell carcinoma (SCC) model also confirmed similar results at 200 MPa HHP for 10 min [21].

We also reported that the 200 MPa HHP treatment did not result in detectable damages in the dermal ECM of normal porcine skin, normal human skin, or human nevus tissues that was assessed by histological or immunohistochemical evaluation, or SEM observation [29]. In addition, the 200 MPa HHP treated dermis was engraftable in the back of a subcutaneously implantable porcine model [19,29]. Based on these cautious results reported previously and also on the results reported in the present study, we applied this novel HHP treatment system to Giant Congenital Melanocytic Nevi and carried out clinical trials [30]. Our EHPT has a limitation about the quality in the extracellular matrices. Severe tumor might have very different ECM from the native skin which cannot be used as skin grafts after EHTP. Tumor should be surgically resected and our EHPT can be applied to cells remaining in the surrounding tissue that causes recurrence.

Another concern associated with this treatment is that the remaining cellular debris may lead to an inflammatory response. In the case of allografts or xenografts, the cellular debris should be thoroughly removed during the decellularization process. However, since our system uses autologous tissue, the level of inflammation induced by cellular debris is acceptably weak, and is comparable to that observed following laser or radiation therapy. In the present study, the tumor cells were completely eradicated by HHP and formed a histiocytic granuloma with calcareous deposition. The necrotic cells induced a phagocytic process of infiltration by macrophages, mononuclear phagocytes, neutrophils and other types of cells. Furthermore, it is reported that the HHP promotes key characteristics of immunogenic cell death, resembling immunogenic chemotherapy, and ionizing irradiation [31]. And HHP generate the dendritic cell-based vaccine [32]. One possible option is the vaccination of autologous inactivated tumor cells. Redical inactivated melanoma cells after EHPT must induce immune reaction as vaccine. The possibility of melanoma vaccine very recently reported at 200 MPa HHP [33]. Then, we believe the reimplantation of the tissue causes the immunogenic reaction toward the cancer.

The main advantages of HHP are the short processing time and that it achieves complete cell killing, regardless of the thickness or

hardness of the tissue, without damaging the matrix. The EHPT process only takes 10 min to inactivate the cells and re-implant the tissue. The whole process can be conducted in the operation room, which minimizes the operation time, the sacrifice of healthy tissue, and the level of stress to which patients are subjected. We also developed a portable HHP device for EHPT that is capable of pressurizing tissue at 200 MPa in the operation room [34].

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

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