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Transient Receptor Potential Vanilloid Subtype 1 (TRPV1)Expression in Bone Marrow-derived Stromal Cells of Rats in vitro

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Abstracts

We studied rat femur bone marrow stroma cells (BMSCs) and microenvironment turnover in capsaicin (CAP)-conditioned media *in vitro*. Proliferation, differentiation and TRPV1 expression at the mRNA level (RT-PCR) of conditioned BMSCs, and pH changes and calcium assay of the conditioned culture media were examined; the obtained data were statistically analyzed. Elapsed time-dependent cell proliferation of BMSCs peaking at day 10 was observed in alkaline phase and 37℃ microenvironment. Although there were no significant differences of proliferation in different conditions on designated experimental days, day 10 was the turning point of proliferation phase where intermittent administration of 25μM CAP resulted in the least amount of cell proliferation than other culture conditions on day 14. The present RT-PCR study revealed expression of TRPV1 mRNA during 3 to 4 days (either day 3 or day 7) after single or intermittent exposure under 25μM CAP. This study elucidated that by intermittent addition of 25μM CAP into the culture medium, the CAP-sensing TRPV1 of the rat BMSCs was activated to significantly mediate Ca^{2+} influx leading to cell death, and the TRPV1 was desensitized between day 7 and day 10 *in vitro*.

Key Words: TRPV1, Stromal Cell, Bone Marrow, Rat

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Introduction

It is known that bone marrow is capable of regeneration because it contains endothelial cells, hematopoietic stem cells and marrow stromal cells (bone marrow stromal cells; BMSCs), which can differentiate into fibroblasts, adipose, reticular and hematopoietic cells, cartilage-like and bone-like colonies *in vitro* [1-6].Mesenchymal stem cells (MSCs) in the bone marrow (bone marrow-derived mesenchymal stem ells) are defined to be undifferentiated cells having potential to differentiate to lineages of mesenchymal tissues [7-12]. Some studies have elucidated that BMSCs are differentiated progenies of the MSCs, and act as a supportive matrix, determining proliferation and cell lineage development. Further, it is found the BMSCs having high adhesive ability to the dish floor differentiated into osteocytic lineages in the present of serum-containing medium [2,3,5,13-16].

Various forms of stimuli, including chemical (i.e., dexamethazone, β-glycerophos phate and mediators) and physical (i.e., mechanical stretch) stimuli, are found to induce cell proliferation, differentiation and transdifferentiation or tissue formation of BMSCs [3,5,7,11,17]. However, when disturbance of the microenvironment of a culture system occurs, cell proliferation would be restricted or/and is followed by either apoptosis or necrosis of culture cells [11,17].

Transient receptor potential (TRP) channel family contains many non-specific (general) lipid-gated cation channels that plays a central role in calcium ion (Ca^{2+}) homeostasis in virtually all cells [18,19]. Some studies on TRP vanilloid (V)-1 receptor (VR1; TRPV subtype 1; TRPV1) have shown that the polymodal nociceptor is a non-selective ligand-gated cation channel can be activated by *N*-vanillyl 8-methyl-1-nonenamide (capsaicin), emperature higher than 43℃, low pH (acidic) environment and other exo- and endo-genous stimuli [19-25]. Many former capsaicin studies were performed *in vivo*, and consisted of capsaicin applications on rat or human skin, which were then examined for degrees of inflammation and pain [24,25]. Treatment with 3 to 30μM capsaicin, which is the main ingredient of pepper, produces a painful, burning sensation by inducing depolarization of TRPV1 nociceptor and activation of the capsaicin-gated ion channel. This then causes the increase of Ca^{2+} concentration in stimulated cells [21,24,26]. Some studies on neural and lung cells cultured in 20μM (48 h) or 50 to 100μM (24 h) capsaicin, have shown apoptosis and cell death related with excessive influx of Ca^{2+}, decrease in endoplasmic reticulum (ER) Ca^{2+} content, accumulation of proteins in ER lumen and load in organelles of TRPV1-overexpressing cells *in vitro* [27,28]. On the other hand, the capsaicin compound is used therapeutically in desensitization of nociceptive pathways [29,30].

TRPV1 was formerly thought to be expressed only in nervous tissue, but recent

studies elucidated that the receptor is also expressed in non-nervous tissues and cells of the bladder, kidney, stomach, spleen and other organs [21,29,31-34]. However, there are no studies that showed the expression of TRPV1 in BMSCs. The present study was undertaken with an aim to examine histological changes and turnover of BMSCs from rat femurs cultured in capsaicin-supplemented conditions, as well as those of the microenvironment *in vitro*.

Materials and Methods

Materials

Cell preparation

Bone marrow samples were sterilely obtained from the femur of rats (5-week-old, male, Slc:Wistar; Shimizu Lab Supplies, Kyoto, Japan). The protocol (No. 06-08002: Culture of rat bone marrow stromal cells) was approved by the Ethics Review Board of Osaka Dental University. The samples were added with Dulbecco's modified essential medium (DMEM; Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% Fetal bovine serum (FBS; Hyclone, Logan, UT, USA), penicillin (100 units/mL; Nacalai Tesque, Inc.) and streptomycin (10 ng/ml; Nacalai Tesque Inc.), and cultured in 10 cm dishes (BD Falcon, Bedford, MA, USA) at 37℃ in a humidified gas mixture containing 50 mL/L CO_2 balanced with air (5% CO_2/air). The bone marrow-derived cells adhered onto the dish were to be the first passage. The cells were grown to sub-confluence and subsequently seeded to be $5X10^3$ /cm^2 cells in DMEM + 5%FBS + penicillin + streptomycin (Medium A), and were sub-cultured to obtain the third passage; medium (Medium A) was changed every 3^{rd}-4^{th} day.

Methods

1. Cell proliferation

The obtained rat BMSCs were seeded $3X10^3$ cells per well in 96 well plates (Asahi Technoglass Co., Chiba, Japan), then cultured under the following five conditions (control: Ⅰ; experimental: Ⅱ, Ⅲ, Ⅳ & Ⅴ with addition of capsaicin) by changing the culture medium every 3 days for 2 weeks. Capsaicin used in the present study was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Condition Ⅰ (control): DMEM + 5%FBS + penicillin + streptomycin = Medium A
Condition Ⅱ: Medium A + 5μM capsaicin = Medium B
Condition Ⅲ: Medium A + 25μM capsaicin = Medium C
Condition Ⅳ: cultured in Medium B for the first 3 days then changed to Medium A
Condition Ⅴ: cultured in Medium C for the first 3 days then changed to Medium A

We examined cell proliferation on days 0, 3, 7, 10 and 14 using Cell Count Reagent SF (Nacalai Tesque). On each experimental day, 10μl of Cell Count Reagent SF was added to each well, and incubated for 1.5 h at 37℃ in 5% CO_2/air, then an absorbance of 450 nm was measured (optical density of 450 nm: OD_{450nm}) The examination of cell proliferation was

performed for 12 wells of each condition.

2. Examination of TRPV1 expression

Some rat BMSCs were seeded 3X10^4 cells per well in 24 well plates (Asahi Technoglass Co.), then cultured under five conditions stated above for two weeks.

Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was performed to confirm TRPV 1 expression at the mRNA level in rat BMSCs cultured under five conditions (conditions Ⅰ, Ⅱ,Ⅲ,Ⅳ&Ⅴ) on days 0, 3, 7, 10 and 14. First -strand cDNA synthesis was performed by using SuperScriptⅢ CellsDirect cDNA Synthesis System (Invitrogen life technologies, Carlsbad, CA, USA). Specific primers were designed for cDNA (obtained on each experimental day 3, 7, 10 and 14) amplification (TRPV1: forward primer: CAAGGCTGTCTTCATCATCC, reverse primer: AGTCCAGTTTACCTCGTCCA, product size: 285 bp; G3PDH: forward primer: ACCACAGTCCATGCCATCAC, reverse primer: TCCACCACCCTGTTGCTGTA, product size: 450 bp; SIGMA-ALDRICH, Tokyo, Japan) following the instructions. First-strand cDNA (2 ng) obtained was diluted to 50μl with a PCR reaction mixture consisting buffer, 1.5 mM MgCl2, 0.2 mM each of dNTP mixture (TOYOBO Co., LTD. Tokyo, Japan), 0.25 units of Blend Taq (TOYOBO Co., LTD), and 10 pmol of each specific primer set (forward and reverse primers). Amplification system was performed in a TaKaRa Thermal Cycler MP (TaKaRa Bio, Shiga, Japan). The amplification system for TPPV1 was conducted at 94℃ (2 min)→ 94℃ (30 secretary)→ 63℃ , and for G3PDH was conducted at 60℃ (30sec) → 72℃ (30sec)X 45cycles→ 72℃ (10min). Amplified samples (PCR products; 5 μl) were analyzed with 1.5% agarose gel electrophoresis at 100 V for 30 min, and the gel was stained with ethidium bromide solution (0.2μg/ml; Nacalai Tesque) for 30min. They were then observed under ultraviolet (UV) light (ATTO Printgraph; ATTO, Tokyo, Japan).

3. The pH changes of the culture medium

The rat BMSCs were seeded (3X10^4 cells per well) in 24 well plates (Asahi Technoglass Co.), and then cultured under five conditions stated above for two weeks. On each experimental day, the pH value of each conditioned medium was examined 3 times using a B-212 twin pH meter (HORIBA Ltd., Kyoto, Japan).

4. Calcium assay

We examined the change of calcium ion (Ca^{2+}) concentration in the media with a DICA-500 QuantiChromTM Calcium Assay Kit (BioAssay Systems, Hayward, CA, USA) according to the manufacture's instructions. The rat BMSCs were seeded 3X10^4 cells per well in 24 well plates (Asahi Technoglass Co .) and cultured under 5 conditions stated above for two weeks. On each experimental day (day 0, 3, 7, 10, 14), each 5μl medium (from either control or experimental group)

was mixed with 200μl working regent and incubated 3 min at room temperature, and assayed with the kit at reading OD of 570-650 nm (peak absorbance at 612 nm: OD612nm) using the Wallac ARVO 1420 multilabel counter. The examination of calcium assay was performed for 6 wells of each condition; the average extracellular Ca^{2+} concentration in each condition was expressed in OD612nm values.

5. Histological examination

Light microscopy

The rat BMSCs were seeded $3X10^4$ cell s/well in 24 well plates (Asahi Technoglass Co.) and cultured under the 5 conditions for two weeks. On each experimental day (day 0, 3, 7, 10, 14), cells of each condition were fixed with 4% parafolmaldehyde, stained with hematoxylin and eosin (H-E), then observed and photographed under an Olympus BK40 light microscopy (Olympus, Tokyo, Japan) following the conventional methods.

Transmission electron microscopy (TEM)

The day 10 specimens cultured in condition Ⅲ were prefixed with chilled 1/4 Karnovsky's fixatives diluted with BPS, washed with BPS, postfixed with 1% OsO_4 solution, washed for several times, dehydrated with ascending series of ethyl alcohol, and then embedded with TAAB 812 epon in culture dishes following the conventional methods. The embedded BMSCs were cut into small pieces, trimmed and then set upon cured epon blocks. The epon-embedded samples were ultrathin-cut with a diamond knife mounted on an LKB 4800 A ULTROTOME I (LKB, Stockholm, Sweden). The specimens were then picked up on 150-mesh copper grids, stained with uranyl acetate (20 min) and lead citrate (10 min), and then examined and photographed with a Hitachi HU-7100 TEM (Hitachi, Tokyo, Japan) following the conventional methods.

6. Statistical analysis

One-factor ANOVA and Kruskal Wallis test for analysis of variance of the obtained data (average) were processed using Excel Statistic 2000 for Windows (Community Information Services, Tokyo, Japan); significant data were assessed by Scheffe's test for post hoc.

Results

1. Cell proliferation

The data were summarized and analyzed for studying cell proliferation on designated days (Figs. 1a & 1b). At 3 days after culture, cell proliferation rate was observed to be the same in all conditions. In conditions Ⅳ and Ⅲ , greater proliferation rates than other conditions was found between day 7 and day 10; a significant increase of 1.5 fold in comparison to day 0 was found in condition Ⅳ on day 10 (Figs. 1a & 1b).

Cell proliferation in all conditions peaked at day 10—approximately 24 h after changing culture media on day 9, and decreased in all conditions between day 10 and day 14 (Figs.

1a & 1b). By comparison with day 0 specimens, we observed active cell proliferation on day 10 (conditions Ⅱ, Ⅲ and Ⅳ), followed by a significant decrease (condition Ⅲ) of cell number thereafter (Fig. 1b). The results indicated that addition of 5μM capsaicin only on first 3 days (condition Ⅳ) significantly induces cell proliferation at day 10 (Figs.1a & 1b).

The present spectrophotofluorometry also suggested that cell proliferation of rat BMSCs on day 10 was significantly affected by addition of capsaicin (conditions Ⅱ, Ⅲ and Ⅳ) relating with elapsed time; day 10 was the turning point of proliferation phase (Figs. 1a & 1b). However, the statistic analysis indicated that variance of cell proliferation was not significantly related with culture conditions on each designated day (Fig. 1a).

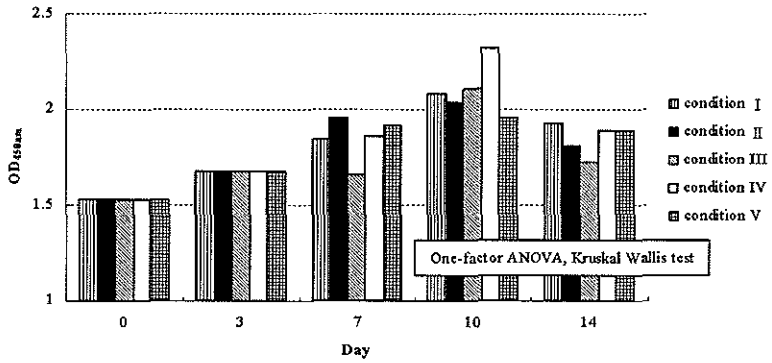

Fig. 1a

Chronological changes of cell proliferation (OD450nm) in conditioned culture media.
Day 10 is the turning point of proliferation phase, however no significant variances are inspected between the five conditions on each designated day.

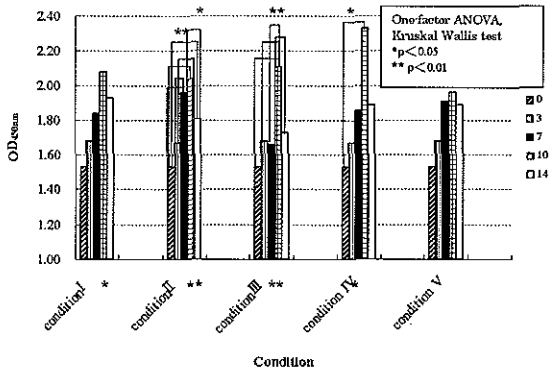

Fig. 1b

Cell proliferation of rat BMSCs in conditioned culture media.
Significant increase ($p<0.05$) of cell proliferation in comparison to day 0 specimens is particularly demonstrated in condition Ⅳ on day 10.

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2. Examination for expression of TRPV1 mRNA

RT-PCR

There was no expression of TRPV1 mRNA in the control group (condition Ⅰ) on all days. Like the control group, conditions Ⅱ (intermittent addition of 5μM capsaicin every 3rd-4th day) and Ⅳ (addition of 25 μM capsaicin on first 3 days) showed no expression of TRPV1 mRNA on all days (Table 1). In contrast, expression of TRPV1 mRNA was observed in condition Ⅲ (intermittent addition of 25 μM capsaicin every 3rd-4th day) and condition Ⅴ (addition of 25 μM capsaicin on first 3 days) on either day 3 or day 7 of experiment (Table 1). However, no expression of TRPV1 mRNA in all five conditions was observed after day 7 (Table 1).

The results suggested that TRPV1 was expressed during 3 days (conditions Ⅲ and Ⅴ) and 24 hours (day 7 of condition Ⅲ) after introducing 25 μM capsaicin into the culture medium.

Table 1

Expression of TRPV1 mRNA on designated days of rat BMSCs in different conditions

	Condition				
	Ⅰ	Ⅱ	Ⅲ	Ⅳ	Ⅴ
Day 0	—	—	—	—	—
Day 3	—	—	+	—	+
Day 7	—	—	+	—	—
Day 10	—	—	—	—	—

(Expression of 285bp Products)

3. pH changes of the culture medium

On day 0 (just after the cells were seeded), the conditioned Ⅱ, Ⅲ, Ⅳ and Ⅴ culture media had higher pH values than that of the control group (condition Ⅰ). The culture medium pH declined towards pH 7.4 for the first 3 days, then recovered to pH= 8.0 to 8.4 on day 4. After that, the culture medium pH declined gradually towards pH 7.4, but never lowered to become an acidic phase in experimental conditions (Figs. 2a & 2b). The present analysis of variance elucidated that there were significant chronological pH changes in condition-Ⅲ, -Ⅳ and -Ⅴ media (Fig. 2b), and the pH differences in conditions Ⅴ and Ⅰ were significant on days 7 and 10 (Fig. 2a).

The results suggests that either 5 μM or 25 μM capsaicin significantly affected pH changes on day 7 and day 10 of culture, and 25 μM capsaicin facilitated alkaline phase of the culture media (Figs. 2a & 2b).

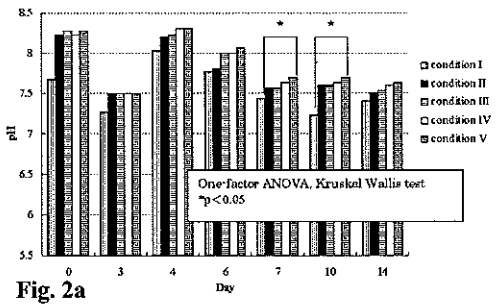

Fig. 2a

Chronological changes of pH in different culture conditions. Significant pH differences ($p<0.05$) are found between media added with capsaicin (experimental group: conditions Ⅱ, Ⅲ, Ⅳ & Ⅴ) and control group (condition Ⅰ) on days 7 and 10. The results indicate that rat BMSCs proliferate in alkaline microenvironment.

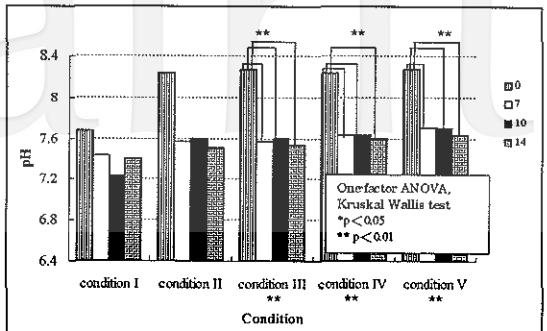

Fig. 2b

Changes of pH in the microenvironment of different conditioned culture media.

Analysis suggests significant pH decrease in conditions Ⅲ, Ⅳ and Ⅴ, and capsaicin facilitates alkaline phase of the microenvironment.

4. Calcium assay

Chronological changes of calcium ion (Ca^{2+}) concentration (represented by OD_{612nm}) of the medium used in both control group (condition Ⅰ) and experimental group (conditions Ⅱ, Ⅲ, Ⅳ & Ⅴ) were studied (Figs. 3a & 3b). The results showed that Ca^{2+} concentration decreased during the day 1, and fluctuated in each condition on days 3, 7 and 10 (Fig. 3a). In comparison with Ⅳ (5 μM capsaicin for 3 days), significant increase of Ca^{2+} efflux in conditions Ⅱ (intermittent addition of 5 μM capsaicin) and Ⅲ (intermittent addition of 25 μM capsaicin) was evident on day 7. In addition, we observed a significant decrease of Ca^{2+} concentration in condition Ⅲ on day 10 (Fig. 3a). The study indicates that, in conditions Ⅲ between day 7 and day 10, the Ca^{2+} concentration of media peaked at day 7 and was followed with a significant decrease (Figs. 3a & 3b).

The results suggests that TRPV1 Ca^{2+} ion channel was possibly activated in accordance with expression of TRPV1 mRNA on day 7 in condition Ⅲ (24 h after additional dose of 25 μM capsaicin), and a significant Ca^{2+} influx of cultured rat BMSCs might occur between day 7 and day 10 (Table 1 & Fig. 3a).

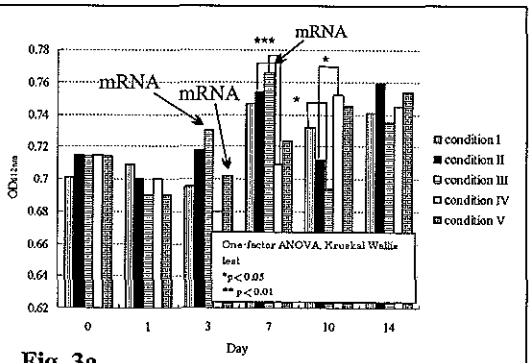

Fig. 3a

Chronological changes of Ca^{2+} concentration (OD_{612nm}) in conditioned culture media.

Arrows showing TRPV1 expression at the mRNA level closely related with addition of 25 μM capsaicin into conditioned -Ⅲ and -Ⅴ culture media. Significant decrease in Ca^{2+} concentration of condition c indicates that intermittent addition of 25 μM capsaicin activates TRPV1 to facilitate Ca^{2+} influx of the proliferating BMSCs between days 7 and 10.

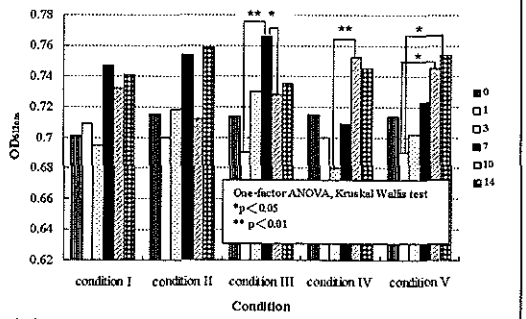

Fig. 3b

Changes of Ca^{2+} concentration in various conditions on designated days. Notice that a significant decrease ($p<0.01$) is found in the condition Ⅲ ECM microenvironment between days 7 and 10.

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5. Histological findings

Light microscopy

On day 3 and 7, there were no histological differences between all conditions; the cultured cells were characterized by a meshwork arrangement (Fig.4a). On day 10, the light microscopy of H-E staining specimens showed that many swollen condition Ⅲ (intermittent addition of 25 μM capsaicin) cells containing brown-stained granular paraplasm had lost the meshwork disposition — a typical cell arrangement usually seen in the condition Ⅰ control group (Fig. 4b). In contrast, the mesh arrangement and sparse distribution of distended cells containing granules were only observed in some parts of day 10 specimens cultured under experimental conditions Ⅱ, Ⅳ and Ⅴ. On day 14, cultures under condition Ⅲ were found to have the least amount of cells, and the brown granular paraplasm present at days 3, 7 and 10 was no longer observed; the rat BMSCs showed no significant cell morphological differences and was rearranged in meshwork for all conditions.

Fig. 4a
Light microscopy of condition Ⅴ BMSCs on day 7 of culture.
The rat BMSCs are characterized by a meshwork arrangement
(N: nucleus; H-E stain).

Fig. 4b
Light microscopy of condition Ⅲ BMSCs on day 10 of culture.
A number of brown-stained granular paraplasm (arrows) are found in a swollen BMSC (N: nucleus; H-E stain).

Transmission electron microscopy (TEM)

Fine structure study showed that BMSCs of condition Ⅲ at 10 days of culture exhibited distended ER and many lysosomes (Figs. 5a & 5b). A number of mitochondria containing needle-like crystallines were also observed in the cytoplasm (Fig. 5b). The histological changes of the cellular membranous structures suggested necrotic cell death of the BMSCs.

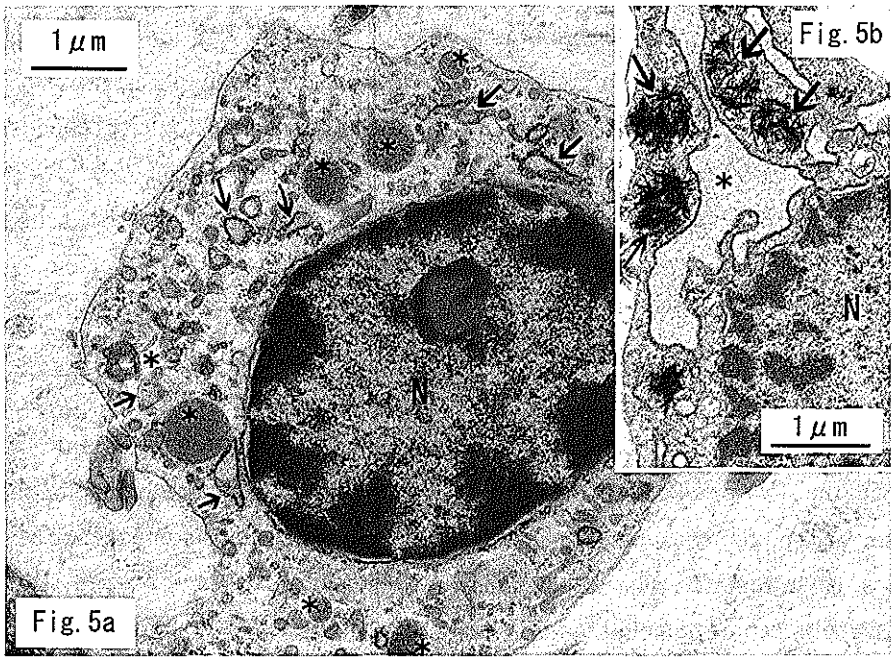

Fig. 5

Transmission electron microscopy (TEM) of condition- Ⅲ rat BMSCs on day 10 of culture (N: nucleus).

5a. A number of lysosomes (＊) of various sizes and distended endoplasmic reticulum (ER; arrows) are found in the cytoplasm. 5b. Distended ER (＊) and mitochondria (arrows) containing needle-like crystalline structures are demonstrated.

Discussion

Many previous studies have asserted that mesenchymal stem cells (MSCs) have the potential to differentiate and regenerate into tissues of mesenchymal origin, and bone marrow stroma is a common pool of multipotent cells (bone marrow stromal cells: BMSCs) [11,35].It is well known that various forms of stimulation and microenvironmental changes are found to play very critical roles in inducing cell proliferation, differentiation, transdifferentiation and survival, and tissue development/formation of BMSCs. Many studies suggest that growth factors, physical and mechanical stimuli, cell density, and cell-to-cell interactions between MSCs contribute to the end product of differentiation associated with the cellular phenotype [3,5,7,11,36].

A study on the change of phenotypes in cultured BMSCs has found that it was associated with the slowing of cell growth and the increase of spontaneous apoptosis [6]. Some studies have reported that capsaicin activated transient receptor potential vanilloid subtype 1 (VR1, TRPV1) receptors of mast cells, bronchial epithelial cells and keratinocytes to release certain growth factors (e.g., cytokines and prostaglandin). Capsaicin up-regulates the expression of growth inhibitors TGF-β 2 and IL-1β , down-modulates the levels of hepatocyte growth factor, insulin-like growth factor-1 and stem cell factor 3 growth stimulators, and suppresses osteoclast differentiation [11,24,37,38].

The sensation of pain is elicited by the activation of sensory nerve endings resulting from various types of chemical, thermal and physiological stimulation. The nociceptors convey stimulus signals to the medulla spinalis, where these signals are interpreted as pain. The TRPV1 that we focus on in this study is a non-selective cation channel playing a pivotal role in systemic osmoregulation and structurally related to members of transient receptor potential (TRP) family. The TRPV1 belonging to the subfamily of TRP ion channel superfamily is activated by noxious heat as well as by luminal stretch, systemic tonicity, chemical and lipid mediators and metabolites [18,29,39]. Additionally, some studies have elucidated that the TRPV1 is a ligand gated, non-selective cation channel which is calcium permeative and a polymodal signal (i.e., stimuli, stretch, tonicity, pain) detector. The TRPV1 is activated and expressed by engagement of capsaicin (a prototypical vanilloid compound activator of TRPV1) and its endogenous ligand, and it is essential in causing the resulting inflammatory hyperalgesia of nociceptive nerves [18-21,27,29,40-42].

There have been many studies indicating that high doses and/or long-term exposure to capsaicin induced the depolarization of nociceptor followed with the consequently intracellular influx of Ca^{2+} ion and desensitization of the TRPV1 reactivity [20-21,41]. After incubation cells with 20 μM (48 h) or 50-100 μM capsaicin (24 h), TRPV1-mediated apoptosis and cell death due to excessive Ca^{2+} influx and load of the cell and intracellular organelles, and reactive oxygen species (ROS)

generation were found in certain TRPV1-overexpressing cells in vitro [27,28,43]. The TRPV1 -mediated apoptosis and necrosis, with excessive Ca^{2+} overload sufficient for mitochondrial damage resulting in inhibition cell proliferation, was controlled by Ca^{2+} uptake via activation of TRPV1 in various cell types [27,39,44]. By other hand, the apoptotic and antiproliferative effects of capsaicin have been elucidated to closely related with p38 mitogen-activated protein kinase (MAPK) activation and ROS generation, which regulates critical steps of the signal transduction cascades and many important cellular events [39,43,45].

TRPV1 has been found to be expressed in the local area of sensory central nerve and spare central nerve system, but recent studies have elucidated that TRPV1 is also expressed in non-nervous tissues/cells of the bladder, utero-epithelium, bowl, lung, kidney, spleen, stomach, heart, neutrophile granulocyte, muscle and mast cells, dendritic and other cells [29,31-34,46-48]. Until now, no studies were available regarding the expression of TRPV1 in cultured BMSCs. There has been a study on the maintenance of peripheral neuron integrity and pain perception. It was reported that nerve growth factor (NGF) stimulated NADPH oxidase pathway to regulate p38-MAPK and induce TRPV1 expression at the protein level but not at mRNA level 37. In the present in vitro study of rat BMSCs, we detected the TRPV1 mRNA in the experimental group (condition V : addition of 25 μM capsaicin for 3 days; condition Ⅲ: intermittent addition of 25 μM capsaicin) on either day 3 or day 7, but not in the control group (condition Ⅰ).

The present study demonstrated a decrease of Ca^{2+} ion in the culture media of conditions Ⅲ, Ⅱ and Ⅰ between day 7 and day 10. The statistical analysis suggests that there were ion channels regulating Ca^{2+} flux in the condition Ⅲ cultures. TRPV1 is a volume-sensitive outwardly rectifier (VSOR) and high calcium transmission non-selective proton channel that is activated at pH threshold of pH 5.9 to 6.0, temperature threshold of 43℃, and 3 to 30 μM capsaicin in various conditions [20,21]. In the present study, we observed time-dependent increase of cell proliferation in alkaline phase particularly in conditions Ⅱ and Ⅲ at 37℃ in 5% CO_2/air. A previous in vivo study has reported a down-regulation of TRPV1 mRNA in cells by certain peripherally derived trophic factors , and the mRNA was not PCR-amplified from all cells showing TRPV1 protein expression [49]. On the other hand, the present RT-PCR study identified TRPV1 mRNA expression in condition V (addition of 25 μM capsaicin on first 3 days) and Ⅲ (intermittent addition of 25 μM capsaicin) on either day 3 or day 7. This therefore indicates differentiation of TRPV1-expression cells from proliferating rat BMSCs in a dose-dependent (above 25 μM capsaicin) manner of the condition-Ⅲ cells at day 7, and it was down-regulated after day 7 in 37℃ and alkaline pH microenvironment in vitro. Proliferation peaking at day 10, followed by a significant decrease in cell

number of condition Ⅲ cultures, implied that day 10 was the turning point of proliferation with a cell turnover resulting in cell death of the BMSCs. Collectively, the findings demonstrated capsaicin-gated TRPV1 activation results in a significant intracellular Ca^{2+} ion flux between day 7 (24 h after additional capsaicin) and day 10. This is in accordance with the expression of TRPV1 mRNA, particularly in condition Ⅲ on day 7. Cell death and significant decrease in cell proliferation between day 10 and day 14 followed the excessive Ca^{2+} ion flux. The TRPV1 was activated and desensitized with intermittent administration of 25 μM capsaic in between days 7-10 and days 10-14.

Activation of alternative pathways of programmed cell death in cells with defective apoptosis has been reported to maintain normal development and homeostasis of cells [50]. Some previous studies have reported that the response to capsaicin (10-200 μmol/L , 24 h) involved apoptotic and delayed cell-death was essentially associated with intracellular Ca^{2+} ion flux [42, 45]. In contrast to non-inflammatory apoptosis, which is a process of genetic regulation of specific cells, necrosis is a typical process associated with some inflammatory/stressful events resulting in cell disruption and fragmentation [51]. Activation of inflammation mediators (e.g., IL-1 β) released by cytosolic and lysosomal secretion has been reported to be associated with cell death [52]. In the present histological studies, we observed distended ER, disrupted mitochondria with needle-like crystalline structures and numerous lysosomes that indicated disturbances in ER loading and calcium homeostasis of the dying BMSCs in condition Ⅲ [28].

In the present study, we observed that a single dose of 25 μM capsaicin elicited the expression of TRPV1 mRNA without necrosis of BMSCs on day 3 of culture. On the other hand, we have attributed necrotic and anti-proliferative effects induced by intermittent administration with addition of 25 μM capsaicin on BMSCs. However, the results suggest that a longer time span for cell culture is required to uncover how adding capsaicin will change cell proliferation and bone marrow stromal cell differentiation. Also, identification of receptors/trophic factors and mechanism to activate and regulate turnover of the TRPV1 channel in the rat BMSCs is what we should confront in future studies.

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辣椒素在體外對鼠骨髓衍生基質細胞(BMSCs)之暫時潛在性辣椒素接受體次型1(Subtypel, TRPV1)的活化作用

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目的：許多不同形態的刺激被發現可在體外引發骨髓基質細胞(BMSCs)的增殖，分化，轉化或組織形成。然而，微小培養環境中的干擾因子限制了細胞增殖，並且導致了培養細胞的死亡。TRPV1是一個非選擇性配位體閘門陽離子通道，可被辣椒素、43°C以上、酸性環境和外在或內生性刺激所活化。當下研究的目的著基於在體外含辣椒素的培養基中，骨髓基質細胞的組織學變化和轉化率(turnover)。

材料及方法：我們研討wistar鼠股骨骨髓基質細胞在體外含辣椒素培養基中的轉化率及培養基微小環境隨之的變化。含添加物培養基中骨髓基質細胞的增殖，分化和以RT-PCR檢測TRPV1作用下產生mRNA的量及含添加物培養基中酸鹼值變化和鈣離子量皆給予檢視，在獲得這些數據後，予以統計分析。

結果：骨髓基質細胞的增殖隨培養天數而變化，在第十天，鹼性與37°C培養基環境下達到最高點。在實驗設計的培養天數，含不同添加物的培養基中，各培養基裡的骨髓基質細胞繁殖情況無顯著差異，而第十天是受培養細胞繁殖的轉折點。結果並顯示出，在第十四天時，與其他培養基比較，間隔添加25 μm辣椒素的培養基中的骨髓基質細胞增殖量最小。而對於TRPV1作用下產生的mRNA所使用的TR-PCR檢測法亦

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顯示出在單次或間隔(3天)添加25 μm辣椒素後，於第三天及第七天可測出這種mRNA的出現。

結論：當下的研究顯示出，在體外試驗中，間隔添加物25 μm辣椒素於培養基中，可使對辣椒素敏感的骨髓基質細胞TRPV1顯著的被活化，並媒介鈣離子匯流入細胞，最後導致細胞死亡。而TRPV1的管道在第七天及第十天時去敏感化。