Fig. 9. The expression of MyoD in muscle-derived primary culture cells by Northern blot analyses. G3PDH mRNA levels obtained by Northern blotting were used for normalization. The expression of MyoD mRNA was not detected after BMP-2 or -4 exposure, and the expression was detected only at 0 and 24 h, and not after 24 h BMP stimulation
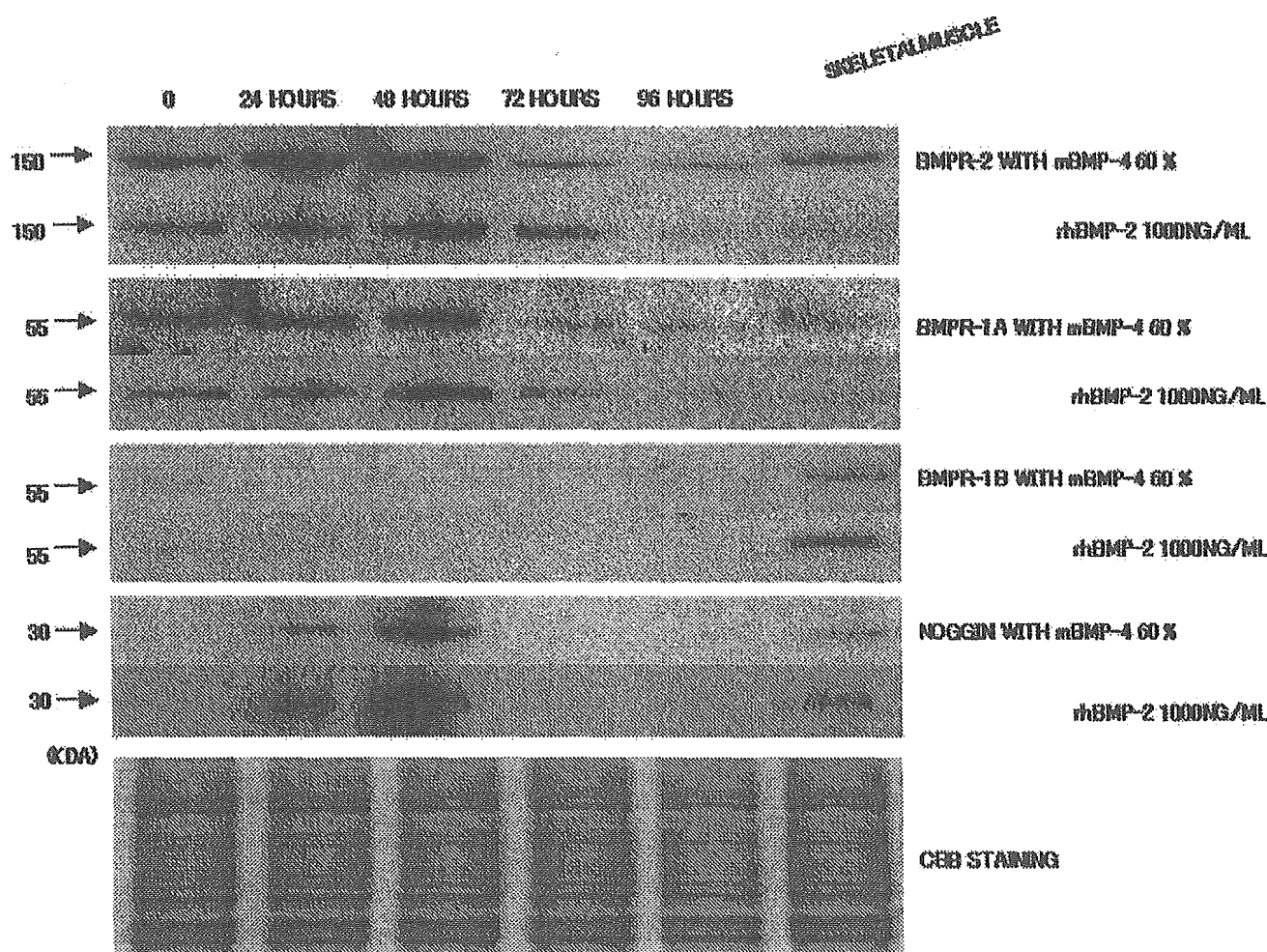

Fig. 10. Western blot analysis of BMPR-1A, -1B, -2, and Noggin after 60% mBMP-4 or 1000 ng/ml rhBMP-2 stimulation in muscle-derived primary culture cells. Equivalent loading and integrity of protein were confirmed by Coomassie brilliant blue staining on the gel (lower panel). Mouse skeletal muscle proteins were used as positive controls. BMPR-1A and -2 were detected at 0 h, induced at 24 h, peaked at 48 h, and then gradually decreased in both 60% mBMP-4 and 1000 ng/ml rhBMP-2 stimulation groups. Expression was greater for BMPR-2 than for BMPR-1A. BMPR-1B was not detectable during any stages in either treatment group. Noggin was not detected at 0 h, was up-regulated at 24 h, peaked at 48 h, and decreased thereafter

In our study, BMPs stimulated them to upregulate the expressions of a bone marker (OC) and cartilage markers (type II collagen and aggrecan, data not shown), but not the muscle marker examined previously. However, it is unclear whether bone and cartilage phenotypes were induced by BMPs in separate cells or in a single cell.

To further understand the potential autoregulatory mechanism in response to BMP, further gene expression

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studies will be necessary. Ultimately, this knowledge may provide new approaches to the regulation of local and systemic bone formation.

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Original article

Effect of low loading and joint immobilization for spontaneous repair of osteochondral defect in the knees of weightless (tail suspension) rats

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Abstract

Background. Mechanical stimulation has a great influence on articular cartilage regeneration. The objective of this study was to clarify the temporal sequences of spontaneous repair of weightless or immobilized joints.
Methods. An osteochondral defect was created in the femoral patellar groove of F344 rats. A tail-suspension procedure was performed to control the mechanical environment of the hindlimbs. The experimental knee joints were classified into three conditions: CONT, normal gait; LLB, low load-bearing; LLI, low load and immobilized. The repair processes up to 4 weeks were evaluated histologically.
Results. The knee defects in the CONT and LLB conditions were repaired to a smooth surface with fibrous tissue and highly developed subchondral bone. The knees in the LLI condition had the lowest reformation rate of subchondral bone, although partial regeneration of hyaline cartilage-like tissue was seen at 4 weeks after the operation. Bulges of fibrous tissue from the defects were observed in the LLI condition.
Conclusions. These results, combined with our previous report, suggest that dynamic compressive strain stimulates regeneration of the joint surface structures. They also suggest that the contact condition of the defect with surface cartilage may play a role in the hyaline cartilage repair.

Introduction

Various reparative therapies were studied for articular cartilage repair. "Microfracture," or "penetration," is a surgical procedure that opens numerous small holes in subchondral bone.[1–4] It is thought that mesenchymal progenitor cells in bone marrow emerge from the holes and regenerate the articular cartilage. This therapeutic method is widely used because of its low-invasive procedure.[5,6] To achieve successful repair of large defects

of cartilage, an autologous osteochondral mosaicplasty technique has been performed clinically.[7] Mosaicplasty involves obtaining small cylindrical osteochondral grafts from the less-weight-bearing periphery of the femoral condyles at the level of the patellofemoral joint and transplanting them in a mosaic-like fashion into a prepared defect site on weight-bearing surfaces. Although mosaicplasty allows relatively short rehabilitation, many investigators have not been satisfied with it because it requires highly advanced technique to reproduce the articular surface congruency, and numerous osteochondral defects are created, sacrificing undamaged cartilage tissue.

Our approach was to develop a new therapeutic method to set up a mechanical environment for hyaline cartilage repair. We showed previously that a functional layer of hyaline cartilage was generated by controlled mechanical movement,[8,9] and other animal studies have demonstrated its use clinically.[10]

We believe that the mechanical environment is as important as the cell source, the scaffold, and the operative procedure. It has been reported that mechanical stimulus influences cartilage matrix synthesis[11–13] and mesenchymal progenitor cell differentiation into the chondrocyte phenotype.[14–16] However, in our knowledge, few in vivo studies have investigated time-sequential changes in articular cartilage regeneration under different mechanical conditions.[17,18] The objective of this study was to evaluate histologically the healing process of articular cartilage defects in rat knee joints that had undergone weightless or immobilization procedures.

Materials and methods

The protocol of this study was approved by the University Committee for Animal Experimentation. Animal experiments were carried out in accordance with
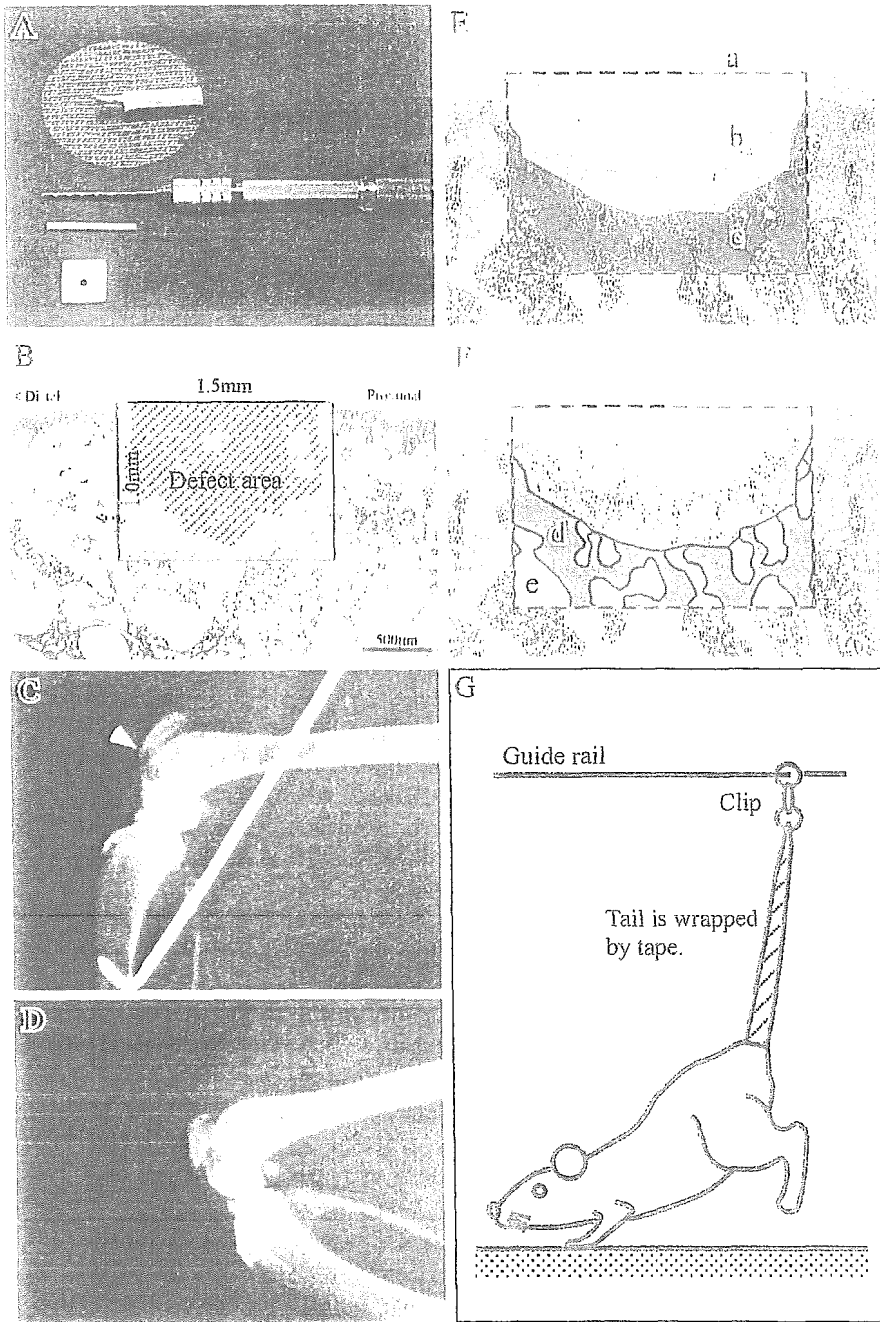
Fig. 1. Full-thickness osteochondral defect (1.5 mm diameter and 1.0 mm depth) was created on the femoral patellar groove. A Hand drill with polyester guide was used to keep the defect depth constantly at 1.0 mm. B Sagittal section of the osteochondral defect with toluidine blue staining. C Radiograph shows the immobilized rat knee joint at 90°. Proximal edge of the defect hole is covered by the patella (*arrowhead*). D Radiograph shows maximum-flexed knee joint. The defect hole was totally covered by the patella at this knee angle. E Defect area (*a*), total refilled tissue area (*b*), subchondral bone area (*c*). Each area was plotted and measured using NIH image software. F Trabecula bone (*d*) and the marrow cavity (*e*). To emphasize the contrast, the trabecula-bone area was intensely painted before the measurement. G Tail-suspension rat model. The rats were elevated off their hind limbs in suspending their tails. The tails were wrapped in surgical tape to attach a clip on the guide rail. The height of the guide rail was chosen so the hindlimbs did not touch the ground

National Institutes of Health guidelines for the care and use of laboratory animals. A total of 36 Fischer 344 rats (female, 13 weeks old, 157–171 g) were used. The rats were anesthetized by intraperitoneal injection of xylazine (10 mg/kg) and ketamine (90 mg/kg). The skin and knee joint capsule were incised using the medial parapatellar approach. A full-thickness osteochondral defect (1.5 mm diameter, 1 mm depth) was created on the femoral patellar groove. The center of the defect was indicated just below the distal end of the patella when the knee joints were held at a 90° degree flexion angle. The defect depth was kept at 1 mm by using hand-

drill with a polyester guide (Fig. 1A). The initial defect area is shown in Fig. 1B. After the operation, rats were randomly classified into two groups: weightless group (*n* = 16) and normal gait group (*n* = 25). The left knee joints of the weightless group were immobilized in 90° of flexion by inserting a Kirschner wire from the tibial tuberosity to the diaphysis of the femur (Fig. 1C). The rats in the weightless group were exposed to a tail-suspension procedure immediately after the operation so their right knee joint was under the low load-bearing condition, whereas the left knee joint was under the low load-immobilized condition. The suspension

technique employed in the present study is a modification of the tail suspension model designed by Morey-Holton,[19,20] where wire insertion was not used, but surgical tape was wrapped around the tail to which a clip was attached (Fig. 1G). Each clip was held by the guide rail running through the ceiling of the cage. These tail-suspension procedures are free from danger, but some psychological and mechanical stress may affect the rat's health judging from their slight weight loss. The test period was chosen to be up to 4 weeks, in view of cruelty to animals.

Animals were killed with an inhaled overdose of diethylether. Knee joints were harvested 1, 2, 3, 4, and 8 weeks after the operation. The distal portion of the femur was removed, fixed in 4% paraformaldehyde at 4°C for 24h, and then decalcified with 10% EDTA. Each specimen was sectioned in the sagittal plane and was evaluated histologically after staining with hematoxylin and eosin (H&E), safranin O and fast green, or, toluidine blue and immunostaining for type I and type II collagen. The total refilled tissue area, subchondral bone area (osseous tissue + marrow cavity), and trabecular bone area were measured using NIH imaging; and the regeneration rate for the initial defect area was calculated (Fig. 1E,F). Statistical comparison for the experimental and control groups was performed using a paired t-test.

Results

Normal gait group (controls)

The control rats started to walk within several hours after the operation. Histologically, 90% of the defect was refilled by newly formed tissue 1 week after the operation. Most of the tissue in the defect was soft tissue, but the number of undifferentiated spindle-shaped mesenchymal cells and the concentration of the extracellular matrix (ECM) showed low density (Fig. 2A). The cell density was increased, and regeneration of trabecular bone was observed 2 weeks after the operation (Fig. 2B). The ECM concentration in the covering soft tissue was increased 3 weeks after the operation. This matrix stained positively with immunostaining for type I collagen. Active regeneration of osseous tissue was also observed (Fig. 2C). The regenerated tissue filled 97% of the defect at 4 weeks after the operation. The regenerated tissue had a smooth surface, and the surface level was almost the same or slightly concave to the surrounding cartilage (Fig. 2D). The defect surface was covered by fibrous tissue, and the reformation of subchondral bone reached almost the same level as the surrounding subchondral bone. The regeneration of hyaline carti-

lage tissue was not observed until 8 weeks after the operation (Fig. 3). Histological features at 8 weeks resembled those seen at 4 weeks.

Low load-bearing group

The regenerated tissue in the low load-bearing group filled 89% of the defect with higher cell density (undifferentiated spindle-shaped mesenchymal cell) than in the control rats 1 week after the operation (Fig. 2E). Regeneration of osseous tissue had already started at this point. The regeneration rate of subchondral and trabecular bone is significantly higher than that of the controls until 2 weeks after the operation (Fig. 5). Increased ECM concentration in the covering soft tissue was observed 2 weeks after the operation. This matrix stained positively with immunostaining for type I collagen. This soft tissue layer maintained a high cell density (Fig. 2F). The regenerated tissue filled 97% of the defect 3 weeks after the operation. The fibrous tissue over the defect surface became thinner, but it connected smoothly with surrounding cartilage (Fig. 2G). Reformation of the osseous tissue reached the same level as the surrounding subchondral bone, and the regeneration rate of the total refilled tissue area was significantly larger than that of the controls (Fig. 5). The histological features at 3 weeks were the same as those at 4 weeks after the operation (Fig. 2H). Regeneration of hyaline cartilage was not observed.

Low load immobilization group

In the low load immobilization group, regenerated tissue filled 87% of the defect 1 week after the operation. The cell density and the ECM concentration was the lowest of all three groups (Fig. 2I). The distal portion of the regenerated tissue projected from the defect. The regeneration rate of subchondral and trabecular bone are significantly higher than that of the controls (Fig. 5). The regenerated tissue seems to have shrunk at 2 weeks after the operation, filling only 76% of the defect. The cell density and ECM content was also still low (Fig. 2J). The regenerative reaction of osseous tissue was weak as well. The regeneration rate of the total refilled tissue area was significantly lower than that of the controls (Fig. 5). The regenerated tissue filled 109% of the defect 3 weeks after the operation. The distal portion of the regenerated tissue projected greatly from the defect (Fig. 2K). Increased cell density, production of ECM, and regeneration of osseous tissue were barely observed. The distal portion of the regenerated tissue still projected from the defect at 4 weeks, and this matrix stained positively with immunostaining for type I collagen. Regeneration of subchondral bone progressed a little, but it never reached the level of the surrounding
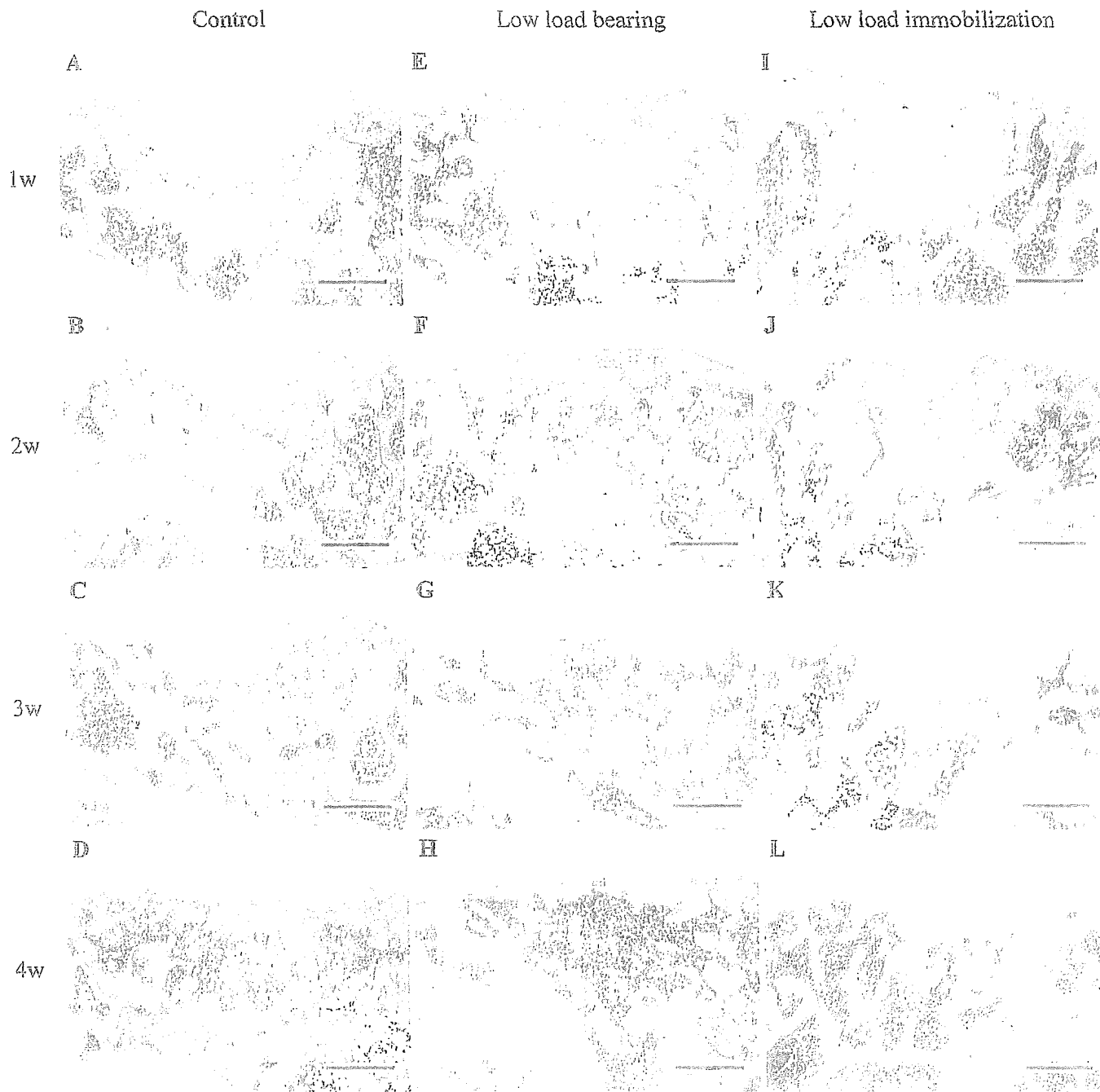
Control   Low load bearing   Low load immobilization

Fig. 2. Sagittal sections show the reparative process in the controls (A–D), low load-bearing group (E–H), low load immobilized group (I–L). *Bars*, 500 μm. H&E, ×10
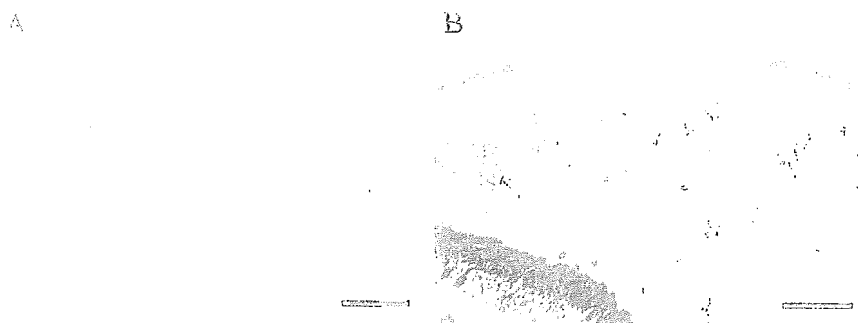

Fig. 3. Sagittal sections of the normal gait control group with hematoxylin and eosin staining (A) or toluidine blue staining (B) 8 weeks after the operation. *Bars*, 500 μm. ×10
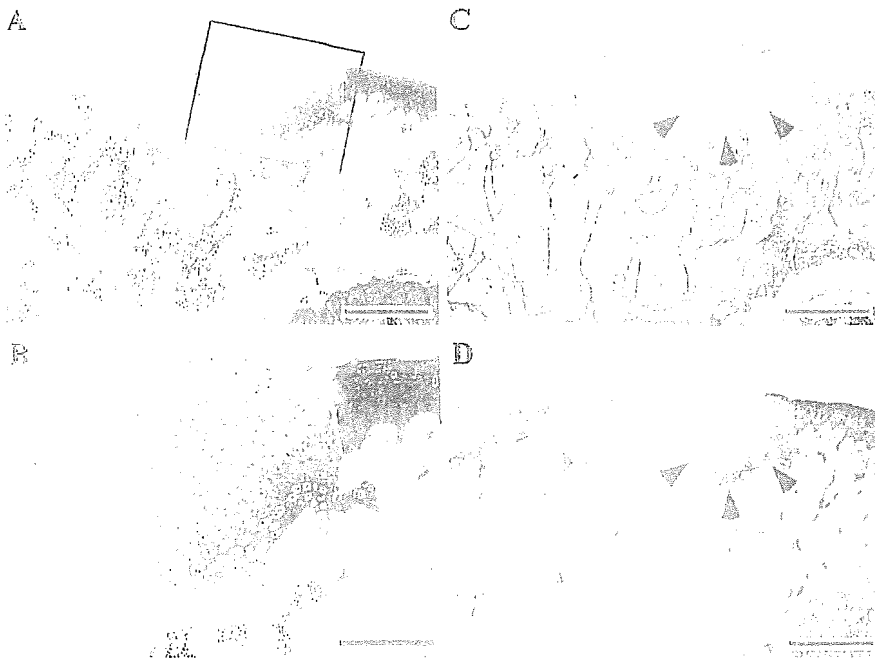
Fig. 4. Hyaline cartilage-like tissue was partially apparent in the low load immobilized group. A Toluidine blue staining in the same section as in Fig. 2L. B Higher magnification of the boxed region in A. C Collagen type I immunostaining in the same section as in A. D Collagen type II immunostaining in the same section as in A. Bars, 500 μm (A,C,D) and 200 μm (B)

subchondral bone (Fig. 2L). The regenerated tissue was covered by thick soft tissue, and the surface level was higher than the level of surrounding cartilage. The regeneration rate of the total refilled tissue area was significantly higher than that of the controls 3 and 4 weeks after the operation (Fig. 5). Interestingly, two of four samples from this group showed neogenesis of hyaline cartilage-like tissue in the proximal half of the defect, as shown in Fig. 4. The ECM of this tissue stained intensely with safranin O and toluidine blue, positively with the immunostain for type II collagen, but negatively with the immunostain for type I collagen.

Discussion

In the rabbit knee joint, it was reported that an articular cartilage defect of less than 3 mm diameter might result in complete or partial repair spontaneously; but if the diameter was more than 5 mm, the defect could not be repaired by hyaline cartilage.[21-24] Hiraki et al. noted that such a geometric difference regarding spontaneous repair of an osteochondral defect was caused by rich or poor recruitment of chondroprogenitor cells from bone marrow.[24]

In the present study, we examined the early temporal sequences of spontaneous repair of osteochondral defects using a relatively small defect. Three mechanical conditions were established using a tail suspension rat model. The repair process and the regenerated tissue constructions were quite varied for each group. The histological results are summarized in Table 1.

Presumably, smooth configuration of the regenerated surface and the regenerative response of mesenchymal progenitor tissues were stimulated by joint motion. Several studies have reported the influence of mechanical stimulus on the regeneration of articular cartilage. Salter and O'Driscoll showed that the repair of articular cartilage was dramatically improved by continuous passive motion.[18,21,25] We also reported that controlled mechanical stimulation induced an articular cartilage-like structure on the surface of osteotomized bone.[8] Cyclic mechanical stress affects the differentiation of mesenchymal progenitor cells to the chondrocyte phenotype.[14-16] These reports mainly showed the influence of optimal mechanical stimulation for articular cartilage regeneration. On the other hand, excessive stress or the immobilized condition has a negative influence (e.g., cartilage destruction, chondrocyte apoptosis, or osteoarthritic changes).[26-28] Espanha et al. showed that articular cartilage defects in the femoral condyles of rats were slightly (or not at all) improved by exercise on a treadmill.[17]

At the beginning of this study we had expected that some hyaline cartilage tissue would appear in the group whose joints were in motion. However, no hyaline cartilage was observed, as shown in Fig. 2E–H. Interestingly, hyaline cartilage-like tissues had partially appeared in the defect of the joint-immobilized group, and it was faced with counter patellar cartilage. Some mechanical or biochemical factors may affect this phenomenon. For example, the patella was thought to apply some dynamic compressive strain on the tissue rather than having a sliding motion in this model. Our
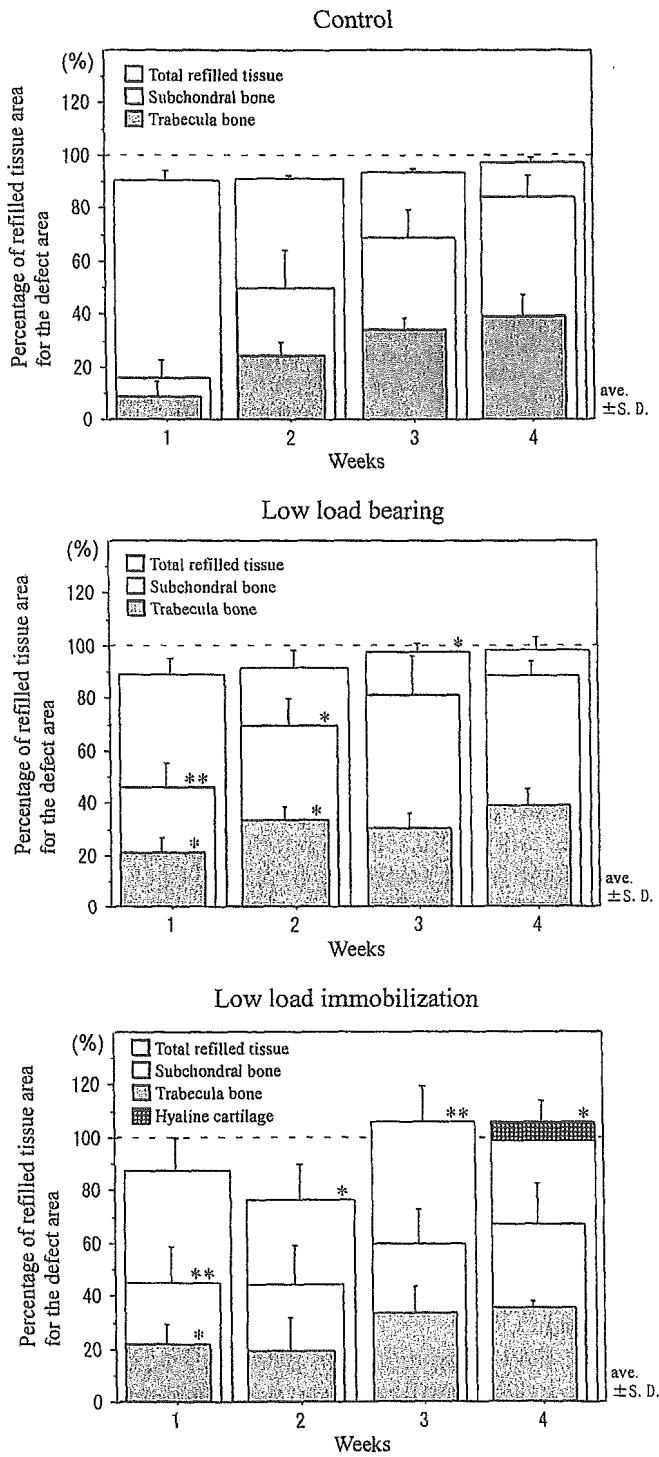
Control

Low load bearing

Low load immobilization

Fig. 5. Percentage of the refilled tissue area for the initial defect area. Subchondral bone area includes trabecula bone and the marrow cavity. Data are presented as the mean ± SD. There were five animals for each period of control. There were four animals for each period of the low load-bearing group and the low load-immobilization group. Hyaline cartilage-like tissue appeared in two samples of low load immobilization group 4 weeks after surgery, and the mean percentage of this tissue is 14.8% ± 4.3% for the initial defect area. *$P < 0.05$ compared with the control group. **$P < 0.01$ compared with the control group

Table 1. Summary of histological examination

Experimental group and weeks	No. of specimens	Percent of refilled tissue for cross section of defect (mean ± SD)			Appearance of hyaline cartilage	Smooth configuration of repaired suface
		Total	Subchondral bone	Trabecula bone		
Normal gait (control)						
1	5	90.0 ± 3.7	15.2 ± 7.3	8.8 ± 5.6	0	0
2	5	90.6 ± 1.3	49.2 ± 14.1	24.1 ± 5.1	0	0
3	5	93.2 ± 1.2	68.6 ± 10.4	34.0 ± 4.5	0	1
4	5	97.0 ± 1.8	83.7 ± 7.9	38.7 ± 8.3	0	5
8	5	100.0 ± 3.2	91.5 ± 1.4	48.0 ± 8.9	0	5
Low load-bearing						
1	4	88.7 ± 6.4	45.5 ± 9.3	20.7 ± 5.6	0	0
2	4	92.9 ± 4.2	69.3 ± 10.3	30.9 ± 1.1	0	2
3	4	97.3 ± 3.4	80.5 ± 14.9	31.2 ± 3.8	0	4
4	4	98.1 ± 4.6	88.3 ± 5.4	39.1 ± 5.9	0	4
Low load-immobilization						
1	4	86.7 ± 12.5	44.6 ± 13.6	21.9 ± 7.2	0	0
2	4	76.1 ± 13.5	43.9 ± 14.5	18.8 ± 12.8	0	0
3	4	108.6 ± 9.7	59.0 ± 13.0	33.5 ± 9.9	0	0
4	4	106.8 ± 6.5	66.8 ± 13.4	35.2 ± 2.6	2	0

previous report showed that a sliding motion with compressive displacement formed a joint-like layer structure, whereas that without any dynamic compressive strain would not. These results, combined with the those of present study, suggest that dynamic compressive stimulation plays an important role in the regeneration of the mechanical structure.

It is hardly necessary to mention that preparation of the mechanical environment is fundamental and indispensable for successful repair of articular cartilage. Effective mechanical stimulation varies in quality and quantity depending on the stage of the healing process of osteochondral defects. Our results suggested that not only the dynamic sliding motion but also some contact condition might play an important role in hyaline cartilage repair.

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ORIGINAL ARTICLE

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The effects of heat on the biological activity of recombinant human bone morphogenetic protein-2

Abstract This study was designed to investigate effects of heat on the bone-inducing activity of recombinant human bone morphogenetic protein (rhBMP)-2. rhBMP-2 samples were heated at 50, 70, 90, or 100°C for 15 min, or 1, 2, 4, or 8 h, or autoclaved at 120°C for 15 min. The bone-inducing activity of the rhBMP-2 before and after heating was assayed in in vivo and in vitro systems. For the in vivo assay, 5 μg rhBMP-2 samples were impregnated into porous collagen disks (6 mm in diameter, 1 mm thickness), freeze dried, and implanted into the back muscles of ddY mice. Three weeks later, the implant was harvested from the host and examined for ectopic new bone tissue by radiography. The new bone mass was quantified by single-energy X-ray absorptiometry. The in vitro activity of the rhBMP-2 was assayed by adding the BMP sample at a concentration of 100 ng/ml to cultures of MC3T3-E1 cells. After 48 h, the alkaline phosphatase activity was measured. After heating at 50° or 70°C, no significant reduction in bone-inducing activity was noted in either in vivo or in vitro assay systems unless the protein was exposed to sustained heat at 70°C for 8 h, based on in vitro assay data. However, heating above 90°C and for longer periods led to a decrease in the biological activity of the rhBMP-2 in a time- and temperature-dependent manner. rhBMP-2 was rendered inactive when exposed to temperatures at or in excess of 120°C.

Key words Heat stability · Bone induction · Molecular structure

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Introduction

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor-β superfamily [1] and possess unique biologic activity that leads to new bone formation [2–4]. BMP-2 is a member of the BMP family, and the human form (hBMP) has been successfully synthesized by the use of DNA recombination technology (rhBMP) for commercial purposes. This protein is now being evaluated as a bone-graft substitute for the treatment of nonunion fractures, bone defect repairs, and spinal fusions [5–11]. In order to use rhBMPs clinically, it is necessary to understand how the molecular structure or bone-inducing activity of BMPs may be modified during transportation, storage, and intraoperative handling. In particular, the stabilization of BMP-retaining devices, or the use of BMP-2 in combination with heat-generating bone cement, are two instances where the stability of the protein would be challenged. Several authors have described the heat resistance of natural crude BMP extracted from the rat, rabbit, and human [12–16], but there has been little work done to look at the effects of heat on the stability and biological activity of rhBMP-2. This study describes how the biological activity and molecular structure of rhBMP respond to varying degrees of heat using in vivo and in vitro assay systems.

Materials and methods

Heat treatment of rhBMP-2

rhBMP-2 was provided by Yamanouchi Pharmaceutical (Tokyo, Japan) in a buffered solution (1 mg rhBMP-2/ml). This was diluted 3-fold (15 μl saline containing 5 μg rhBMP-2) and heated at 50, 70, 90, or 100°C for 15 min, or 1, 2, 4, or 8 h using a heat block (Dry Thermo Unit, Taitec, Saitama, Japan), or autoclaved at 120°C for 15 min. The biological activity of these heat-treated rhBMP-2 samples was then evaluated in in vitro and in vivo experiments to examine

changes in the biological activities of the rhBMP-2. As a control, rhBMP-2 that had not been exposed to heat was used in the assays.

Cell culture

A mouse osteoblastic cell line MC3T3-E1 was obtained from the RIKEN Cell Bank (Tsukuba Science City, Japan). These cells were seeded at a cell density of 3×10^5 cells/100-mm plastic dish and cultured with α-minimal essential medium (Gibco, Grand Island, NY, USA) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Gibco) at 37°C in an incubator with 95% humidified air and 5% CO_2.

Induction of alkaline phosphatase (ALP) by rhBMP-2

The biological activity of the rhBMP-2 was assayed based on the observation that BMP is capable of inducing expression of ALP in a dose-dependent manner. ALP activity is an early marker of osteoblastic differentiation [17]. The rhBMP-2 samples, heated as described above, were added to the MC3T3-E1 cells at a final concentration of 100 ng/ml. After 48 h, the osteoblastic cells were washed twice with phosphate-buffered saline, scraped from each well into 0.3 ml 0.5% NP-40 containing 1 mM $MgCl_2$ and 10 mM Tris (pH 7.5), and sonicated twice for 20 s with a sonicator (model W-220; Wakenyaku, Kyoto, Japan). The cell lysates were then centrifuged for 10 min at 3000 r.p.m., and the supernatants were used for the enzyme assay. ALP activity was assayed using the method of Kind–King [18] and a test kit (Alkaline Phospha K, Wako Pure Chemical Industries, Osaka, Japan) with phenylphosphate as a substrate.

The enzyme activity was expressed in King–Armstrong (K–A) units normalized to the protein content of the sample. The protein content was determined with a bicinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL, USA) using bovine serum albumin as the standard. Each heat-treated rhBMP sample was measured in three separate dishes, and the ALP activity from each group was expressed as mean ± SD.

Preparation of BMP-containing collagen disks

In order to evaluate the influence of heating on the bone-inducing activities of the rhBMP-2, a classical in vivo experimental system of ectopic bone induction in mice was utilized. In this system, BMP was combined with collagen and implanted into muscle, whereupon an ectopic ossicle was elicited if the BMP was biologically active. In order to deliver the BMP, commercially available porous collagen sheets made from bovine Achilles tendon (Helistat Integra Life Sciences, Plainsboro, NJ, USA) were cut into round strips (6 mm in diameter, 1 mm thick), soaked in the sample solutions containing 5 µg of either the heated or nonheated control rhBMP-2, freeze-dried, and stocked at −20°C until implantation into mice.

Assay for the bone-inducing activities of heat-treated rhBMP-2

One hundred and seventy-six male ddY mice, 5 weeks of age, were purchased from Nippon SLC Co. (Shizuoka, Japan). The mice were housed in cages with free access to food and water for 1 week before the start of the experiment. Prior to surgery for implantation of the collagen/rhBMP-2 composite collagen disks, the mice were anesthetized with diethylether. The BMP-retaining pellets were implanted into the left dorsal muscle pouches (one pellet per animal). Twenty-two groups of mice (8 mice in each group) were implanted with collagen disks containing 5 µg rhBMP-2 that had never been heated (control), or heated at 50, 70, 90, or 100°C for 15 min, or for 1, 2, 4, or 8 h, or autoclaved at 120°C for 15 min. The mice were fed for 3 weeks and then killed. The implants were harvested and examined for ectopic new bone formation in situ by radiographic and histological methods. X-rays of the harvested tissues were taken with a soft X-ray apparatus (Sofron Co., Tokyo, Japan). In order to quantify the ectopically induced bony mass, the bone mineral content (BMC, mg) of each ossicle was measured by single-energy X-ray absorptiometry using a bone mineral analyzer (DCS-600; Aloka Tokyo, Japan). For the histology, the harvested tissue mass from each group was fixed in neutralized 10% formalin, defatted in chloroform, decalcified with 10% ethylenediamine tetraacetic acid, and embedded in paraffin wax. Sections of 5 µm thickness were cut, stained with hematoxylin–eosin, and observed under a light microscope.

All procedures for the animal experiments were carried out in compliance with the guidelines of the Institutional Animal Care Committee of Shinshu University.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

In order to examine the effects of heat on the rhBMP-2 molecules, heated or nonheated samples of the protein were electrophoresed on a SDS-PAGE slab gel. Five micrograms of nonheated rhBMP-2 or protein heated at 90°C on a heat block or at 120°C in an autoclave for 2 h were dissolved into 20 µl sample buffer solution (0.5 µM, pH 6.8 Tris-HCl buffer solution/0.2% SDS/10% glycerol/0.01% bromophenol blue) with or without 100 mM dithiothreitol (DTT, a disulfide-bond reducing agent) and boiled for 5 min. Each sample was run on a SDS gel at 40 mA for 60 min. The gel was then stained with Comassie brilliant blue and destained in 10% acetic acid solution.

Statistical analysis

Quantitative data were expressed as the mean ± SD. Differences between control and experimental groups were considered to be statistically significant at $P < 0.05$ using the Kruskal–Wallis H-test with Bonferroni correction.

Fig. 1. The influence of heat treatment on rhBMP-2-induced alkaline phosphatase (*ALP*) activity of MC3T3-E1 cells. Data are means ± SD of three culture wells. A significant difference from the control is indicated as * ($P < 0.05$)
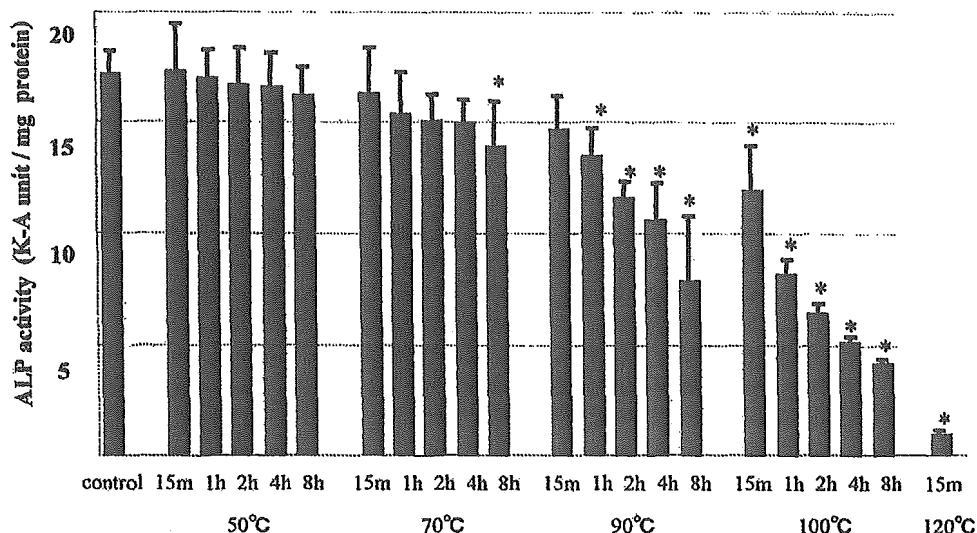

Fig. 2. Soft X-ray photograph of an ossicle formed at 3 weeks after implantation. A typical implant from each group is shown. *Scale bar* = 6mm. There is no difference in the trabecular pattern within ossicles from each group. However, a reduction in size was observed over 90°C in a time-dependent manner. In the groups heated at 100°C for 4h, at 100°C for 8h, and at 120°C for 15min, there was no firm mass around the implant location. Thus, we decided not to collect tissues from these areas for further analysis (softex and bone mineral content)
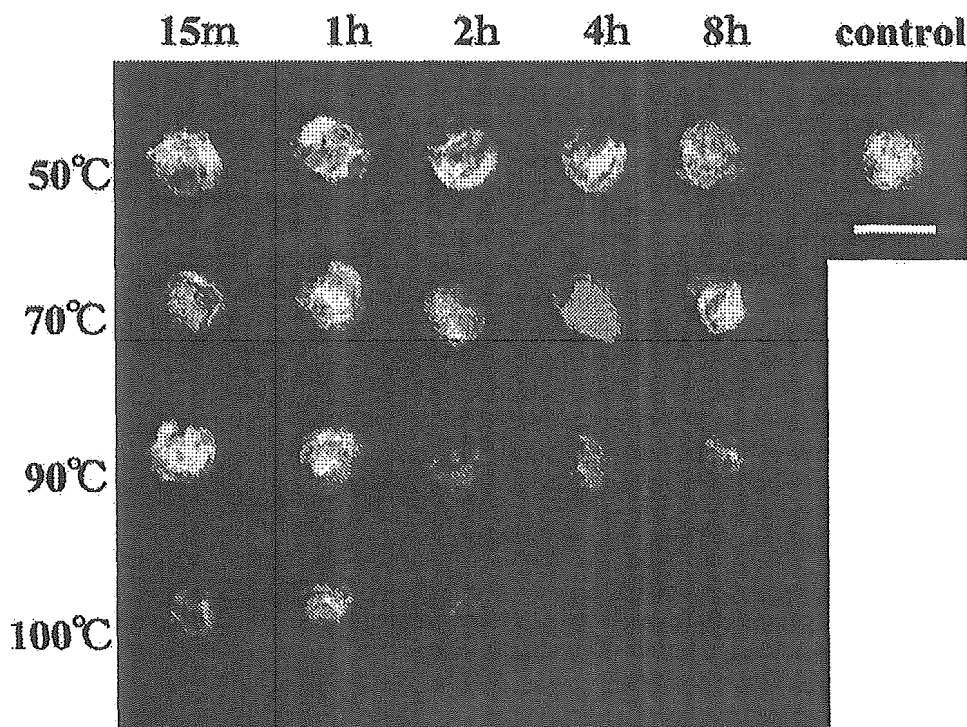
Results

Effects on ALP expression by heated rhBMP-2

Figure 1 shows the effects of the heat treatment on the ALP-inducing activity of rhBMP-2 in MC3T3-E1 cells. ALP-inducing activity was preserved at 50°C even after the rhBMP-2 was heated at this temperature for 8h. A notable reduction in the ALP-inducing activity of rhBMP-2 was seen using protein samples heated at 70°C for 8h. At 90°C, a significant reduction of activity was noted by heating for 1h or more in a time-dependent manner. By heating

at 100°C, the time-dependent reduction was profound. The ALP-inducing activity was reduced to less than 1 unit/mg protein when the rhBMP-2 was heated at 120°C for 15min.

Change in the bone-inducing activity of rhBMP-2 by heating

Figure 2 shows representative ossicles elicited in mice within a 3-week period by implanting collagen disks impregnated with 5μg rhBMP-2 heated at 50, 70, 90, or 100°C for 15min, or for 1, 2, 4, or 8h. Ossicle size tended to become
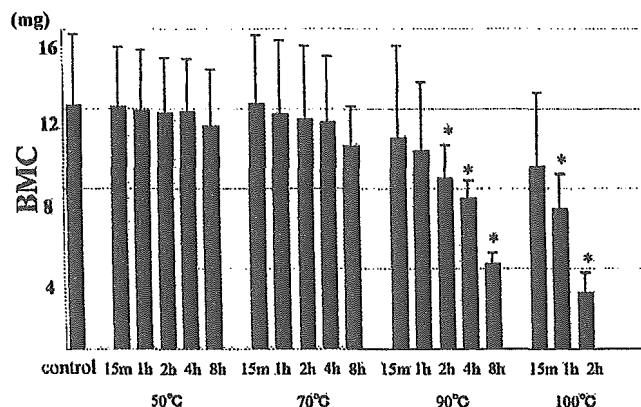
Fig. 3. The bone mineral content (BMC) of the tissues harvested at 3 weeks after surgery. Data are means ± SD of 8 samples. A significant difference from the control is indicated as * ($P < 0.05$). There was no significant difference between the BMC of the 50°C and 70°C groups and the control group. However, the mean BMCs of groups heated at 90°C for 2, 4, or 8 h and the one heated at 100°C for 1, or 2 h ($P < 0.01$) were significantly lower than that found in the control group. In the groups heated at 100°C for 4 h, at 100°C for 8 h, and at 120°C for 15 min, we found no evidence of new ectopic bone formation, as described in the legend to Fig. 2

Fig. 4. Photomicrograph of the tissue harvested at 3 weeks after implantation (heated to 50°C for 8 h) (H&E, ×200). Normal bone histology with hematopoietic marrow and bony trabeculae was observed

small when the rhBMP-2 was treated at 90°C for 2 h or more. After treatment for more than 4 h at 100°C or for 15 min at 120°C, the bone-inducing activity of rhBMP-2 was lost.

Figure 3 shows the mean BMC values of the ossicles from each of the groups. The mean (±SD) BMC of the control group was 12.2 ± 3.56 mg. In the group treated at 50°C for 8 h, the mean BMC was 11.2 ± 2.81 mg, which was not significantly lower than that of the controls. In the group with protein heated at 70°C, the mean BMC in the group treated at 70°C for 15 min was 12.2 ± 3.45 mg. The BMC in the 70°C group showed a downward trend, but no statistically significant difference was observed when compared with the control group. Similarly, in the group using protein heated at 90°C, the BMC of the harvested ossicles decreased with an extension of the heating time. The BMC of ossicles from the groups treated at 90°C for 15 min or 90°C for 1 h were not significantly different from those of the control group. However, the mean BMC values of the groups treated at 90°C for 2 h (8.48 ± 1.68 mg), at 90°C for 4 h (6.98 ± 1.06 mg), or at 90°C for 8 h (4.26 ± 0.53 mg) were significantly reduced when compared with those of the control group ($P < 0.01$). Again, in the group treated at 100°C for 15 min, the BMC was less than that of the controls, although there was no significant difference between the values. The mean BMC of the groups treated at 100°C for 1 h (6.96 ± 1.71 mg) or at 100°C for 2 h (2.80 ± 0.99 mg) were significantly lower than those from the control group ($P < 0.01$).

Histological analysis of the ossicles from all groups revealed normal bone histology with hematopoietic marrow and bony trabeculae (Fig. 4).
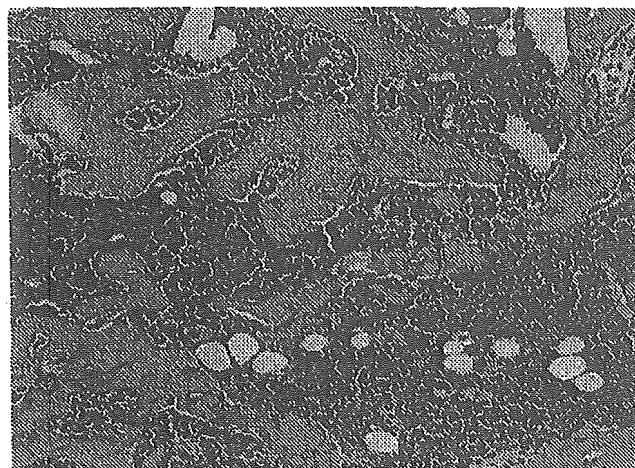
Changes in the SDS-PAGE profile of rhBMP-2 by heating

The SDS-PAGE profiles of rhBMP-2 before and after heating at 90 or 120°C are shown in Fig. 5. The original rhBMP-2 showed three dark bands at around 30-k Daltons (kD) and two light bands below 20 kD without DTT (lane 1). With DTT, the 30-kD bands became lighter and the lower molecular-sized bands became darker (lane 4). By heating at 90°C for 2 h, in the absence of DTT the 30-kD bands became a little indistinct and two lower sized bands disappeared (lane 2), and with DTT the 30-kD bands became lighter and the lower bands became darker (lane 5). By heating at 120°C for 2 h, in the absence of DTT the 30-kD bands remained although the three bands became indistinct (lane 3), and with DTT both of the 30-kD bands and the lower bands were lost (lane 6).

Discussion

BMPs (BMP-2, BMP-7) with potent bone-inducing activity have been successfully produced and developed for clinical use through DNA recombinant technology. Examples of clinical applications include the repair of damaged bone, the reconstruction of bone defects resulting from trauma, and resection of bone tumors. In terms of a successful clinical outcome, the bone-inducing activity of rhBMP-2 must be able to withstand the conditions associated with transportation and storage. In addition, rhBMP has to survive sterilization and the high temperatures encountered when implants are used in close contact with curing bone cement. Previous studies have qualitatively described the stable character of natural BMP based on the ability of crude insoluble BMP-retaining decalcified bone matrix to induce ectopic new bone. Nakanishi et al. [12] reported that rabbit bone-derived BMP extracts elicited ectopic bone after
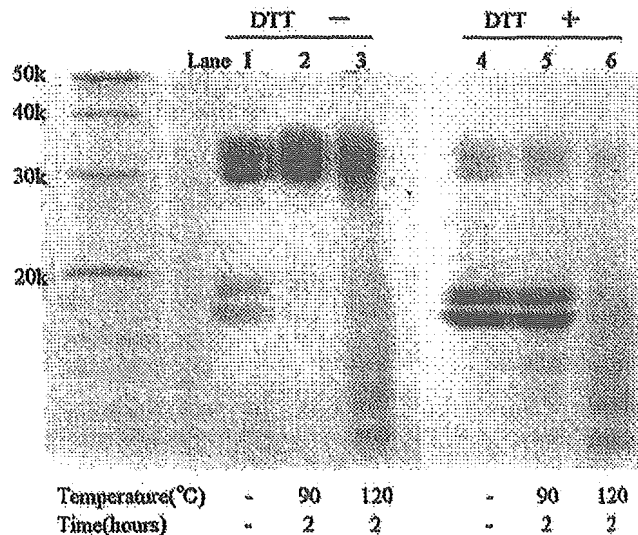
Fig. 5. SDS-PAGE profiles of rhBMP-2 before (*lane 1*) and after (*lane 2*) heating at 90°C and at 120°C (*lane 3*) for 2 h. *Lanes 4, 5*, and *6* show the respective samples under reducing conditions in the presence of dithiothreitol (DTT, a disulfide-bond reducing agent). The original rhBMP-2 showed three dark bands at around 30k Daltons (kD) and two light bands below 20 kD without DTT (*lane 1*). With DTT, bands at 30 kD became lighter and the lower bands became darker (*lane 4*), which indicates that the dimers were reduced to monomers. By heating at 90°C for 2 h in the absence of DTT, the major 30-kD bands became a little indistinct, and the two lower-sized bands disappeared (*lane 2*). With DTT, bands at 30 kD became lighter and the lower bands became darker (*lane 5*), indicating that the bands at 30 kD without DTT maintained a dimeric structure. By heating at 120°C for 2 h, the original size of rhBMP-2 was maintained, although the three distinct bands were lost (*lane 3*). With DTT, both of the original 30-kD bands and those lower molecular-sized bands were lost (*lane 6*), indicating complete denaturation of the original configuration of the molecules

heating at 70°C for 10 min. However, the bone-inducing activity was lost when the sample was heated at over 100°C for 20 min. Some reports have also described similar results using crude BMP extracts as the bone-inducer in rodent models [13,14]. One report demonstrated that BMP activity was retained in a rabbit model after heating the matrix protein at 170°C for 10 min or at 140°C for 30 min [15].

In this study, the bone mineral content of the rhBMP-2-induced ossicles was used to evaluate the bone-inducing activity of the heat-treated rhBMP-2 in an in vivo system. In addition, the ability of rhBMP-2 to induce ALP activity in osteoblastic cells in culture was used as a model to confirm the results from the in vivo experiments. The ALP-inducing activity of the rhBMP-2 was little affected by heat treatment up to 70°C for 8 h or up to 90°C for 1 h, but then decreased gradually with increasing temperature and time. The ALP-inducing activity was eventually lost after heating the rhBMP-2 at 120°C, a treatment that denatured the molecular structure of the rhBMP-2 as shown on a SDS-PAGE profile. These results were in accordance with the in vivo results, and suggest that rhBMP-2 is largely stable in nature. The use of autopolymerising polymethylmethacrylate (PMMA)-based bone cement is the most common method

chosen for prosthetic component fixation in total joint replacement surgery. During the polymerization of PMMA, high peak temperatures are reached. The temperature peak ranges from 48°C to 105°C at the bone–cement interface, and from 80°C to 124°C in the cement. The exposure time over 50°C is reported to be between 30 and 400 s [19]. Thus, the data from the present study suggest that contact with curing bone cement would not affect the bone-inducing activity of rhBMP-2. Sterilizing by autoclave (120°C) would effectively inactivate the biological action of this protein, but sterilizing by ethylene oxide gas (60°C for 3 h or 40°C for 6 h) would not, as reported previously [20].

The heat-stable character of BMP may be derived from its molecular structure. It is known that the bioactive BMP-2 molecule consists of a homodimer which is essential for its biological activity. The homodimer consists of two 114 amino acid monomers with 7 cysteine residues that form three intramolecular disulfide bonds, with one intermolecular disulfide bond forming a cysteine knot [21]. The heat-resistant nature of the BMP might be explained by the cysteine-knot formation, which is known to contribute to the structural stability of the protein.

The biological activity of the rhBMP was reduced following heat denaturation at 90°C for 2 h as evaluated in an in vivo assay system, and for 1 h in an in vitro assay system. At these higher temperatures, the reduction in rhBMP-2 activity was temperature- and time-dependent. In order to visualize the heat-dependent changes in molecular structure, changes in the SDS-PAGE gel profile after heating were observed. As previously described, the rhBMP-2 molecule has a molecular size of around 16 kD with one N-glycosylation site, and it forms a homodimer which has biological activity [6–8,22]. Therefore, the three bands at 30 kD in the control lane on the SDS-PAGE with no disulfide bond reduction indicated dimeric BMP-2 molecules with 2, 1, or no sugar chains, respectively (see Fig. 5). Further, the two faint bands below 20 kD were monomers with or without glycosylation because of their similarity to the molecular sizes of the DTT-reduced rhBMP-2 monomer. By heating at 90°C for 2 h, in the absence of DTT the dimer bands became a little indistinct and the monomer bands were lost, which indicated complete deformation of the rhBMP-2 monomer. With DTT, the dimer bands became lighter and the monomer bands became darker. These changes most likely reflect partial deformation of the original three-dimensional configuration of the rhBMP-2 dimers, which corresponds with the partial loss of bone inducing activity. A higher temperature (120°C for 2 h) made the dimer bands more indistinct and eventually degraded the structure of the monomers, as seen on DTT-reduced SDS-PAGE, with the resultant loss of biological activity.

In conclusion, the biological activity and molecular structure of rhBMP-2 were shown to be fairly resistant to heat treatment. In considering the potential clinical uses of rhBMP-2, the stable character of the protein suggests that it would not need to be cooled during transportation or storage. In addition, it was found that contact with heated material such as curing bone cement would not affect the

bone-inducing activity of the rhBMP-2. However, the auto-claving of rhBMP-2 causes this protein to lose its biological activity based on the results of the present study.

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家庭用品に使用される化学物質の細胞毒性：
平成9～16年度対象化学物質の結果

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Cytotoxicity of Chemicals used in Household Products: 1997－2004

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Cytotoxicity of Chemicals used in Household Products: 1997-2004

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The cytotoxicities of chemicals used in household products were evaluated using a neutral red (NR) uptake assay. The chemicals tested during 1997 − 2004 were rubber additives (accelerators, antioxidants and retarders), solvents, plasticizers and biocides, such as antimicrobials, fungicides, preservatives used in paints, paper, wood and plastic products. The cytotoxicity potential of each chemical was classified by determining the concentrations inducing 50% reduction of NR uptake into Chinese hamster fibroblast V79 cells compared to control (IC50). In vivo eye irritancy of each chemical was estimated by the IC50 value. Most biocides tested showed strong cytotoxicity and had a high probability of inducing strong eye irritation.

Keywords: cytotoxicity, household product, biocide, rubber additive

緒言

家庭用化学製品の製造にあたってはできるだけ安全性の高い化学物質を適切な量で用いられることが望まれる．しかし，現状ではメーカーは用いている化学物質について十分なデータを持ち合わせておらず，健康被害防止についてリスク管理等の十分な評価が行われているとは言えない．厚生労働省では「有害物質を含有する家庭用品の規制に関する法律」に基づき，規制基準を設定し安全対策を講じている．さらに健康被害の未然防止策として，使用頻度，事故例の有無及び文献調査を行い，毎年3〜4種の化学物質を選定して安全性評価の試験検査をしている．試験項目として，細胞毒性試験，変異原性試験，連続投与試験，生殖／発生毒性試験，感作性試験などがある．

我々は，これらの試験項目のうち細胞毒性試験を平成3年度から担当してきた．試験方法としてはニュートラルレッド（NR）法を用いている[1]．NR法はドレイズ眼刺激性試験[2]の結果と相関があるとされているが，我々も界面活性剤等で試験した結果，NR細胞毒性試験とドレイズ眼刺激性試験とが定量的にも相関があることを確かめている[3,4]．本研究では平成9年度から16年度に選定した28品目の細胞毒性試験の結果について報告する．

実験方法

1. 試験物質

当概年度に試験品目として選定された化学物質をTable.1に示した．これらのうち，試薬メーカーから購入できるものは市販品を用い，そうでないものはメーカーから使用原体を供与された．それぞれの化学物質は精製することなくそのまま試験に用いた．

2. 細胞

チャイニーズハムスター由来線維芽細胞V79細胞を用いた．細胞はウシ胎児血清を10%含有させたEagle's MEM培地を用い，培養フラスコで増殖させた．

3. 細胞毒性試験

既報に従って行った[3,4]．試験時，トリプシン−EDTA溶液を用いて培養フラスコからV79細胞を回収し，リン酸緩衝液を加えて1000 rpmで5分間遠心して洗浄後，5％ウシ胎児血清を含有させたEagle's MEM培地（FBS−MEM）に浮遊させた．96穴プレートに9000個/100 μl/wellの割合で細胞を入れ，37℃，5％炭酸ガス培養器中で24時間培養後，上清を除き，種々の濃度の被験物質を含有させたFBS−MEMを200 μlずつ加えた（公比2の割合で5段階，1濃度あたり4穴）．対照（コントロール）は被験物質を含有しない新鮮培地を加えた．また，試験の妥当性と再現性を確かめるため，zinc diethyldithiocarbamate（ZDEC）を標準対照物質として用い，毎回同時に試験した．24時間培養後，上清を除

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Table.1　Result of cytotoxicity test

Chemical	Abbreviation	CAS No.	IC50 (μg/ml) [a]	
Biocide				
N-n-Butyl-1,2-benzisothiazolin-3-one	BBIT	4299-07-4	6.6	Str
1-Bromo-3-ethyoxycarbonyloxy-1,2-diiodo-1-propene	BECDIP	77352-88-6	1.7	Str
	BNPD	52-51-7	7.7	Str
Cycloacetamide	CAA	79-07-02	47.5	Mo
p-Chlorophenyl-3-iodopropargylformyl	CPIP	29772-02-9	6.2	Str
4,4'-Dimethyl-1,3-oxazoline	DMO	51200-87-4	46.0	Mo
N,N'-Hexamethylene-bis(4-carbamoyl-1-decylpyridinium bromide)	HMBCDPB	Unknown	7.0	Str
Hiba oil	HO	Unknown	16.2	Str
	IPBC	55406-53-6	2.9	Str
Methylene-bis(thiocyanate)	MBTC	6317-18-6	1.08	Str
10,10'-Oxy-bis(phenoxyarsine) [b]	OBPA	58-36-6	4.2	Str
4-Chloro-3-methylphenol (*p*-Chloro-*m*-cresol)	PCMC	59-50-7	89.9	Moo
4-Chloro-3,5-dimethylphenol (*p*-Chloro-*m*-xylenol)	PCMX	88-04-0	37.0	Mo
2,3,5,6-Tetrachloro-4-(methylsulfonyl)pyridine	TCMSP	13108-52-6	1.32	Str
2-(Thiocyanomethylthio)benzothiazole	TCMTBT	21564-17-0	1868	Wea
4,4'-Tetramethylene-bis(4-carbomoyl-1-decylpyridium bromide)	TMBCDPB	Unknown	6.8	Str
Zinc naphthenate	ZnN	12001-85-3	59.0	Mod
	ZPT	13463-41-7	0.42	Str
Plasticizer				
Di-n-butyl sebacate	DBS	109-43-3	1600	Wea
Diethyl sebacate	DES	110-40-7	1085	Wea
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate	TMPDIB	6846-50-0	170	Mod
2,2,4-Trimethyl-1,3-pentanediol monoisobutyrate	TMPMIB	25265-77-4	233	Mod
Rubber accelerator				
Zinc butylxanthate	ZBX	150-88-9	5.6	Stron
Zinc isopropylxanthate	ZIPX	1000-90-4	18.3	Stron
Rubber antioxidant				
N-(1-Methylheptyl)-*N'*-phenyl-*p*-phenylenediamine	MHPPD	15233-47-3	14.4	Stron
Octylated diphenylamine	ODPA	101-67-7	4500	Weak
Styrenated diphenylamine	SDPA	17796-82-6	146	Mode
4,4'-Thio-bis(3-methyl-6-*tert*-butylphenol)	TBMBP	96-69-5	0.58	Strong
Rubber retarder				
N-(Cyclohexyl)thiophtalimide	CTP	17796-82-6	16.6	Strong
Solvent				
Diethylene glycol mono-*n*-butyl ether acetate	DEGEA	121-17-4	1500	Weak

[a] The value represents the mean of 3 experiments.

[b] 2% in propylene glycol.

き，NR を 50 μg/ml の濃度で含有させた FBS-MEM 200 μl ずつを加えてさらに3時間培養した．上清を除き，2.5％ホルマリン－1％ CaCl₂ 溶液を 280 μl 入れて1分間静置して細胞を固定，洗浄し，上清を捨てた後，1％酢酸－50％エタノール溶液を 100 μl 加えて，細胞内に取り込まれた NR を抽出した．540 nm における吸光度を測定し，各物質それぞれの濃度について対照群の吸光度値に対する％を計算してこれを縦軸に，試験濃度を対数で横軸にとったグラフにプロットした．各試験物質について吸光度を50％にする濃度（IC50）を求めた．実験は3～4回繰り返し行い，その平均値を得た．

結果と考察

　細胞毒性試験はドレイズ眼刺激性試験法の代替法とし

ての有用性が多くの実験者によって確認されており [1,3] NR 取り込み能を指標とした方法もその1つとして」られている．生物試験では同じ操作をしても試験ごと若干の結果の変動が起こることが経験的にわかって り，今回のように毒性強度をクラス分けするには一定基準を置いて試験することが望ましい．医療機器のた ドラインでも毎回標準物質を置くことが推奨されて る [6]．そこで，本研究でも毎回の試験では ZDEC を標物質として同時に実施することにより，試験法の感度 再現性を確かめた．Table.2 には毎年の ZDEC の IC50 を示した．その結果，変動が少なくほぼ一定の値が得 れることから，IC50 値から判断する試験物質のクラ 分けが各年度とも同一の基準でされていることが確認 きた．我々はこれまで，350 μg/ml 以上の IC50 値を示

Table.2　Reproducibility of IC50 value for zinc diethyldithocarbamate

Experimental year	IC50 (μg/ml) [a]
1997	1.77
1998	1.62
1999	1.51
2000	1.73
2001	1.23
2002	1.58
2003	1.93
2004	1.48
(Mean ± SD)	1.60 ± 0.21

[a] IC50 was the concentration that reduce the absorbance by 50% of control.

物質を弱い細胞毒性物質で眼刺激を起こす可能性がほとんどない，35～350 μg/ml を中程度の細胞毒性物質として眼刺激性が誘発される可能性がある物質，35 μg/ml 以下を強い細胞毒性物質で明らかな眼刺激性を起こす危険性があるとした[4]．本研究でもこの判定基準をそのまま用いることにした．

試験物質はバイオサイド18種，可塑剤4種，ゴム加硫促進剤2種，ゴム老化防止剤4種，スコーチ防止剤1種，溶剤1種である（Table.1）．可塑剤と溶剤の細胞毒性強度は強くはなく，ゴム老化防止剤に用いられるものは添加される物質によって強度に大きな差があった．今回試験したxanthate系ゴム加硫促進剤はこれまで試験したcarbamate系と同様に強い細胞毒性を示すことがわかった．

近年，抗菌性をうたった家庭用品が多く出回っており，これらに使用される抗菌剤は日本防菌防黴学会によってまとめられている[7]．こうした薬剤には農薬として用いられてきたものを用途転換したものも多く含まれる．そのため，使用対象・方法が変わることによって皮膚等との接触頻度が増え，健康被害が起こる可能性がある．こうしたことから抗菌剤を始めとしたバイオサイドの安全性について確認する必要性が強くなり，そのため，この期間の試験対象物質としてはバイオサイドが半分以上を占めた．バイオサイドは製品の微生物汚染を防ぐ薬剤を総称しており，欧州では消毒剤，防腐剤，生物抑制剤，その他の4つの分類に分けており，さらに木材，繊維，ポリマー等防腐対象とするものによって小分類されている[8]．日本では，防かび剤（木材用，その他），防腐剤，防藻防かび剤，抗菌剤，防虫忌避剤等で使われる物質が相当するが，1つの化学物質が複数の効果を示したり，使用先が多岐にわたったりすることから，ここではバイオサイドでまとめて記載した（Table.1）．最も強いものはzinc bis(2-pyridylthio-1-oxide)，別名ジンクピリチオンで，シャンプーなどにふけ防止剤として使用されている．以下，TPN，TCMSPと続くものの，試験したほと

んどのバイオサイドがstrongと判定された．殺菌性ということを考えるとある程度強い細胞毒性を示すことは予想できる．一方，TCMTB は最も IC50 値が低く，TCC も細胞毒性は弱かった．IC50 値から判断するとバイオサイドのほとんどが眼刺激性を起こす可能性が高いとした．

National Institute for Occupational Safety and Health の Registry of Toxic Effects of Chemical Substances (RTECS) で各バイオサイドの実験動物に対する眼刺激性を調査したところ，PCMXは 100 mg で moderate の反応が表れたとしている[9]．しかし，細胞毒性試験で最も毒性の弱かった TCMTBT は 30％含有物 100 mg で moderate との反応を示し，in vitro 試験との相違が認められた．ZPT と BNPD は投与量については記載があるが反応性の報告はなかった．他の物質については in vivo 眼刺激性データを示す報告は認めなかった．本細胞毒性試験法と皮膚刺激性との間に相関性はほとんどないが[4]，動物実験での皮膚刺激性について参考に調べたところ，BNPD，BIT及びTMTBT は mild～moderate と判定され，OBTAはモルモットに対し連続塗布したところ，severe な反応を示したとある[9]．Draize試験は実験者によって適用量が違うこと，1用量だけの試験で表れた反応強度だけをもとにしている場合がほとんどであり，このような用量反応関係を示さない試験での刺激性強度の判定を細胞毒性試験の結果と単純に比較するのは難しいのかもしれない．

欧州は，バイオサイド製品の上市に関する指令を採択し，抗菌剤の統一した法律管理をとることとし，既存化学物質の調査を行った後，審査対象物質を選定し，安全性試験のデータの提出を求めている[8, 10～12]．我が国でも繊維業界等では使用できる抗菌剤の表示と加工について自主基準が作られているものもあるが[13～16]，多くの物質は家庭用品について適用した場合の安全性についてはほとんど検討されていない．ここで示したように，使用されるバイオサイドには強い細胞毒性を起こすものがあり，使用法の制限，配合量と溶出量との相関性など，これからも健康被害を防止するための検討が必要と思われる．

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