a greater extent than EGF [14]. Midkine was identified as a retinoic acid-inducible differentiation factor in an embryonic carcinoma cell line, and with the pleiotrophin-/heparin-binding growth-associated molecules it constitutes a unique family of heparin-binding proteins, which was isolated from rat brains as a neurite outgrowth-promoting protein expressed during the developmental stage of rapid axonal growth [15,16]. These reports suggest that glucosaminoglycans and heparin-binding proteins might be effective in therapy for disorders of the central nervous system and neurogenesis. Thus, we examined the effects of sulfated hyaluronan (SHya), a novel glucosaminoglycan, on astrocyte activity in this study.

Heparin has the largest number of sulfated groups in mammalian tissues, but the number of sulfated groups varies. We synthesized sulfated hyaluronan (SHya) by sulfating hyaluronan, a non-sulfated glucosaminoglycan; these synthesized SHyas have regular numbers of sulfated groups (Fig. 1). In previous studies, we demonstrated the effects of SHya on cell proliferation and differentiation. SHya increased the adhesion molecules *N*-cadherin and connexin43 on the mRNA level in cultured rat calvarial osteoblasts [17]. SHya also enhanced human keratinocyte differentiation and altered mRNA levels of Wnt, Notch1, and Notch3, which play important roles in cell proliferation and differentiation [18]. These studies suggested that SHya could similarly affect glucosaminoglycans that enhance FGF-2 and midkine activities. Therefore, we examined the effects of SHya on normal human astrocytes (NHA) activities, such as proliferation, morphological change, and production of astrocyte trophic factors. In addition, we compared the effects of SHya with that of FGF-2 on astrocyte activities and hypothesized the functional mechanism of SHya.

2. Materials and methods

2.1. Materials

Sulfated hyaluronan (SHya) was synthesized in our laboratory by the method reported previously [18,19]. The molecular weight of SHya was 2.0×10^5, and the degrees of substitution (D.S.) of SHya were 0.4 and 1.0, as determined by the chelate titration method (Fig. 1) [20]. Normal human astrocytes (NHA) were purchased from Cambrex Bio Science Walkersville, Inc. (MD, USA), and NHA culture medium (ABM) (ANG bred kit) was from Sanko Junyaku Co., Ltd., Tokyo, Japan. Human recombinant FGF-2 was purchased from BD Biosciences (Franklin Lakes, NJ, USA).

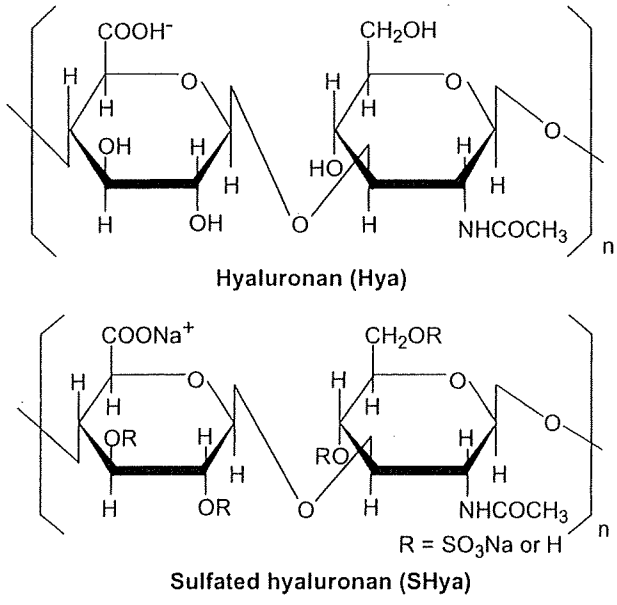

Fig. 1. Structures of hyaluronan (Hya) and sulfated hyaluronan (SHya). SHya is composed of Hya and sulfated groups. The molecular weight of SHya is 2.0×10^5, and the degrees of substitution of SHya were 0.4 and 1.0.

2.2. V79 colony assay

Cytotoxicity was examined using V79 cells by a colony assay following the "Guidelines for Basic Biological Tests of Medical Materials and Devices – Part III: Cytotoxicity tests [21]". Chinese hamster fibroblast V79 cells were obtained from Japanese Cancer Research Resources Bank (Tokyo, Japan) and grown in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin in a 37 °C humidified atmosphere of 5% CO_2 and 95% air. For the assay, 50 V79 cells/ml were seeded on 24-well plates in Eagle's MEM supplemented with 5% FBS and 1% penicillin–streptomycin. After 24 h incubation in the 37 °C humidified atmosphere of 5% CO_2 and 95% air, 1 ml of the medium with SHya0.4 (0.1, 1, 10, 50, 100 μg/ml), SHya1.0 (0.1, 1, 10, 50, 100 μg/ml) or without (control) was added to each well, and the cells were cultured for 7 days. The colonies formed were fixed with 10% formalin solution and stained with 5% Giemsa solution. The number of colonies on each well was counted, and the efficacy of SHyas was calculated as a ratio of the number of colonies in the sample to that in the control. The data were expressed as an average of four wells, and the procedures were performed in duplicate.

2.3. NHA MTT assay

Effects of SHya on mitochondrial activity of NHA were measured using a microtiter tetrazolium (MTT) assay. NHA cells were extracted from a human fetus at 18 weeks gestation. The basic culture medium was ABM medium supplemented with 5% FBS and recombinant human epidermal growth factor, insulin, GA-1000, ascorbic acid, and L-glutamate (ANG bred kit). NHA were seeded into 24-well plates at a density of 1×10^4/well in ABM medium at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Stock solution of 100 μg/ml SHya1.0 was made directly in ABM medium. After 1 week culture with 0.1, 1, 10, or 100 μg/ml of SHya0.4 or SHya1.0, the medium in each well was replaced with 300 ml of fresh medium containing 6 ml TetraColor ONE reagent (Seikagaku Corporation, Tokyo, Japan). After 2 h, the absorbance at 450 nm/630 nm was measured using a plate reader. The data were expressed as averages of five wells, and the procedures were performed in triplicate.

2.4. Proliferation of NHA cells

NHA were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in ABM (control), ABM supplemented with 10 μg/ml SHya0.4, or ABM supplemented

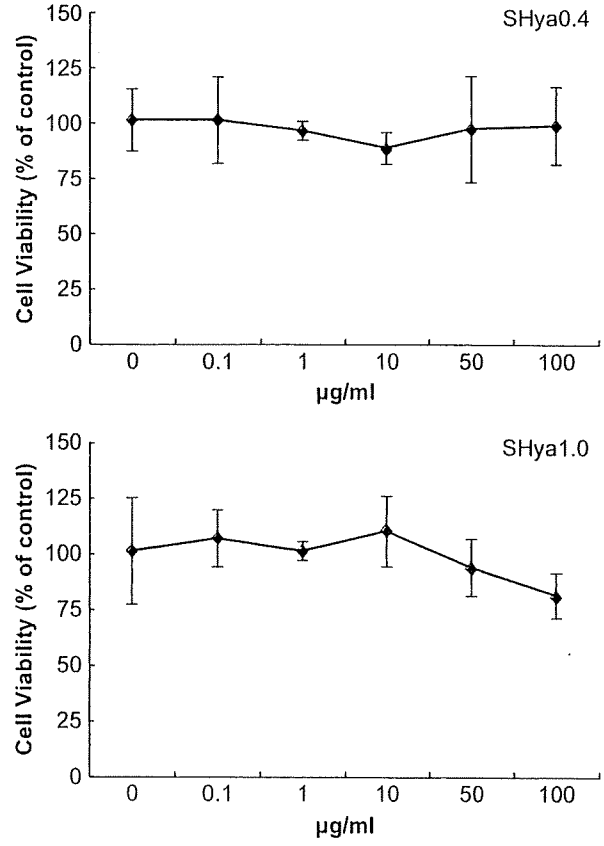

Fig. 2. Viability of V79 cells cultured with SHyas. V79 cells were treated with SHya0.4 or SHya1.0 and cultured for 7 days. Data are expressed as mean ± SD ($n = 4$).

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with 10 μg/ml SHya1.0; all three media were supplemented with or without 10 ng/ml FGF-2. Stock solutions of 100 μg/ml SHya and solutions of 1 mg/ml FGF-2 were made directly in ABM medium. The medium was exchanged for a fresh one every 2 days, and NHA were passaged every 7 days. The NHA proliferation data were expressed as averages of three wells.

2.5. Immunocytochemical methods

NHA cells were cultured in ABM (control) or ABM supplemented with 10 μg/ml SHya1.0, and both media were supplemented with or without 10 ng/ml FGF-2 for 10 days. Plated cells were fixed in 4% paraformaldehyde for 30 min and rinsed in phosphate-buffered saline (PBS). Fixed cultures were blocked in 10% blocking reagent (Block Ace Powder; DS Pharma Biomedical Co., Ltd., Osaka, Japan) with 0.3% Triton X-100 and incubated with primary antibodies to GFAP (polyclonal, 1:500, neuron glial cell marker sampler kit; Millipore, Tokyo, Japan) at room temperature overnight. After incubation with the primary antibody, cultures were rinsed in PBS and incubated with fluorescein-conjugated goat anti-rabbit IgG (1:500, Alexa Fluor 488 goat anti-rabbit IgG; Molecular Probes, Eugene, OR, USA) at room temperature for 1 h. After incubation with the second antibody, cultures were rinsed in PBS and incubated for 30 min in 300 nM 4′,6-diamidino-2-phenyl-indole dihydrochloride (DAPI) (Molecular Probes) to stain nucleic acid at room temperature. After incubation, cultures were rinsed in PBS again, and their cells were observed by fluorescence microscopy.

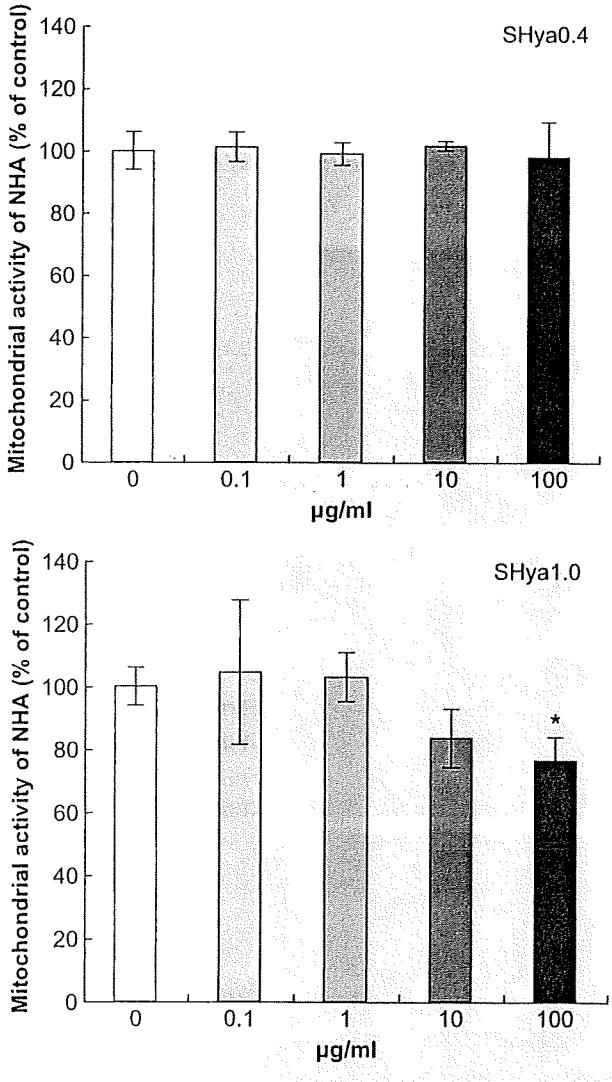

Fig. 3. Mitochondrial activity of NHA cultured with SHyas. Astrocytes were cultured with 0.1, 1, 10, 100 mg/ml SHya0.4 or SHya1.0 for 7 days, and mitochondrial activity was measured by MTT assay. Data are expressed as mean ± SD ($n = 3$). Experimental data showed significant difference from the control group (*, $p < 0.05$).

2.6. Scrape-loading and dye transfer (SLDT) assay

The SLDT technique was performed by the method of El-Fouly et al. [22]. NHA were cultured in ABM for 3 weeks. The medium was changed to a fresh medium containing SHya 48 h before the assay. NHA cells in a confluent monolayer in 35-mm culture dishes were rinsed with Ca^{2+}, Mg^{2+} phosphate-buffered saline [PBS(+)], and the culture dishes were filled with 0.1% Lucifer Yellow (Molecular Probes) in PBS(+) solution and immediately scraped with a sharp scalpel. After 5 min incubation at 37 °C, cells were washed four times with PBS(+), and the extent of the dye influx into cells was monitored using a fluorescence microscope equipped with a type UFX-DXII CCD camera and a super high-pressure mercury

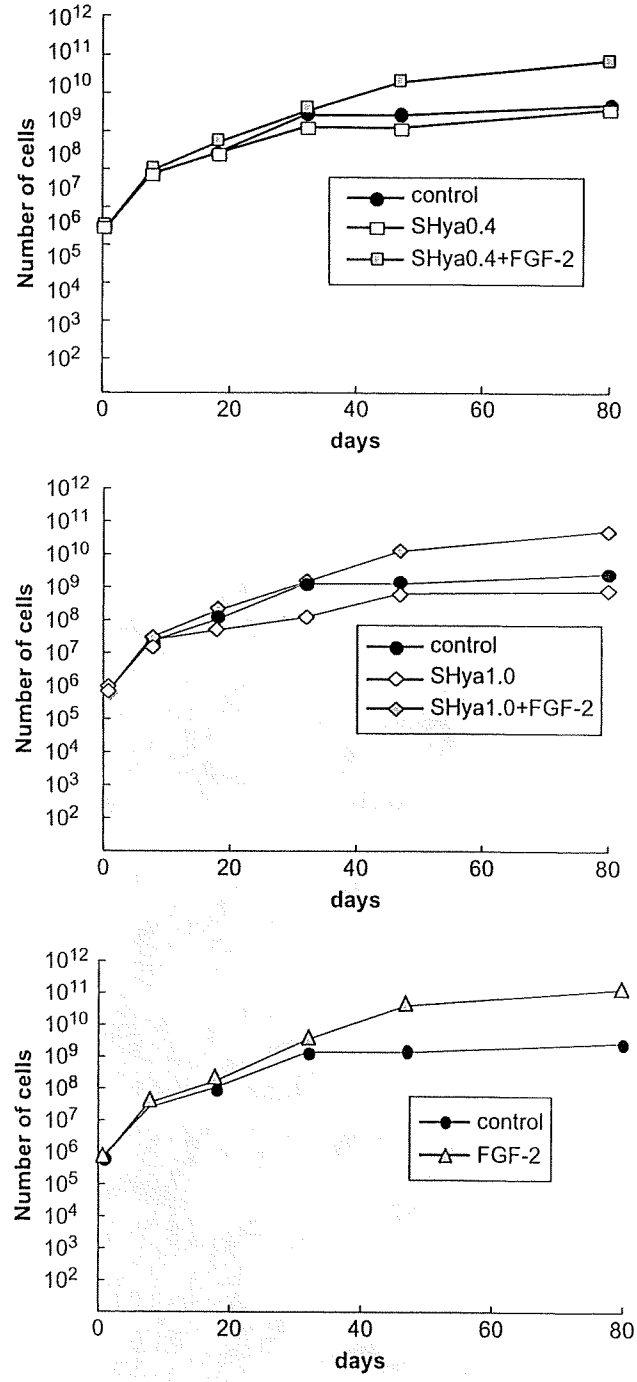

Fig. 4. The effect of SHya, FGF-2, and SHya with FGF-2 on the proliferation of cultured NHA. The concentrations of each regent were 10 μg/ml SHya0.4, 10 μg/ml SHya1.0, and 10 ng/ml FGF-2. Data are expressed as mean ± SD ($n = 3$).

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lamp power supply (Nikon Co., Tokyo, Japan). The data were expressed as averages of four wells.

2.7. Expression of neurotrophic factor mRNA

NHA were seeded into six-well plates. When NHA were semi-confluent, the medium was changed to a fresh medium containing SHya. NHA were incubated 24 h at 37 °C, and total RNA was extracted using an RNeasy Mini Kit (Qiagen Sciences, Germantown, MD, USA). First-strand cDNA was synthesized from total RNA using a first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). Midkine and BDNF mRNA were measured quantitatively by a real time PCR light cycler and kit (Roche, Mannheim, Germany). Primer sequences for amplification were 5′-GGCTTGACATCATTGGCTGA-3′ and 5′-CCTCCAGCAGAAAGAGAAGAGG-3′ for BDNF, 5′-AGCCAAGAAAGGGAAGGGAAA-3′ and 5′-TGATTAAAGCTAACGAGCAGACAGA-3′ for midkine. For measurements of FGF-2, NGF, IGF-1 and β-actin mRNA, a human mRNA quantitative 2 step RT-PCR primer set (Search-LC, Heidelberg, Germany) was used. The RNA preparation and real time PCR in the present study were performed in duplicate.

2.8. Statistical analysis

Data for individual groups were expressed as mean ± SD, and two-way ANOVA was performed. The Tukey–Kramer test was used to analyze differences between the control and other groups. In all cases, $p < 0.05$ was considered significant. Results were expressed as mean ± SD.

3. Results

3.1. V79 colony assay

Treatment of SHya0.4 did not alter V79 cell viability. Treatment with 100 μg/ml SHya1.0 tended to decrease V79 cell viability, but the difference was not statistically significant (Fig. 2).

3.2. Mitochondrial activity of NHA

Treatment of SHya0.4 did not alter mitochondrial activity of NHA. Treatment with 100 μg/ml SHya1.0 decreased mitochondrial activity of NHA significantly (Fig. 3).

3.3. Proliferation of NHA cells

NHA cells increased in number for about 30 days (Fig. 4); thereafter, they increased slightly. NHA cell body was enlarged throughout in vitro culture (Figs. 5–7).

Treatment with 10 μg/ml SHya0.4 hardly affected NHA proliferation. Treatment with 10 μg/ml SHya1.0 delayed NHA proliferation, but NHA proliferation had almost caught up that of the control by 50 days. Treatment with 10 ng/ml FGF-2 increased NHA proliferation for 50 days, and cell numbers increased markedly compared with control. In addition, treatment with a combination of SHyas and FGF-2 also increased NHA proliferation without the delay effect of SHya1.0. However, NHA proliferation due to combinations of SHya0.4 or SHya1.0 and FGF-2 was lower than that due to either FGF-2 alone (Fig. 4).

3.4. Morphological change of NHA

Treatment of NHA with 10 μg/ml SHya1.0, 10 μg/ml SHya0.4 with 10 ng/ml FGF-2, or 10 μg/ml SHya1.0 with 10 ng/ml FGF-2 transformed cells into a stella morphology (stellation) in 5 days of culture (Fig. 5). In 10 days of culture, NHA cell bodies were enlarged

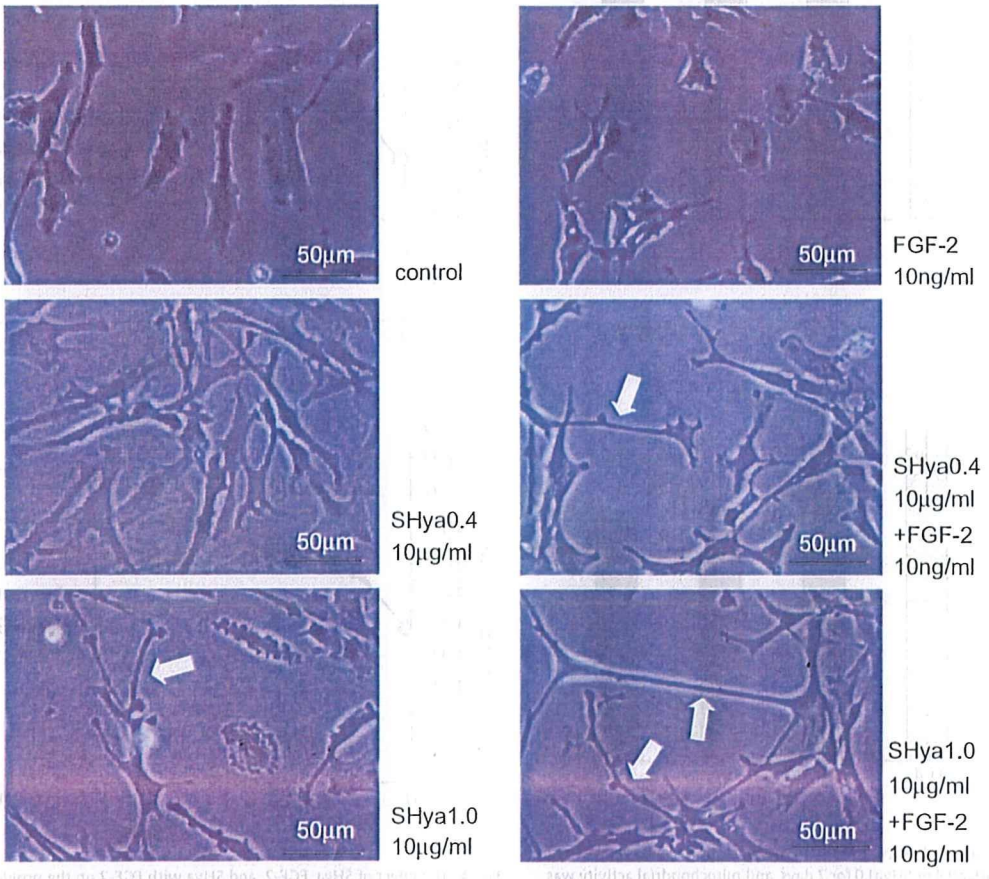

Fig. 5. Effects of SHya, FGF-2, and SHya with FGF-2 on morphological changes in cultured NHA over 5 days. The concentrations of each reagent were 10 μg/ml SHya0.4, 10 μg/ml SHya1.0, and 10 ng/ml FGF-2. The arrow shows stellation of NHA.

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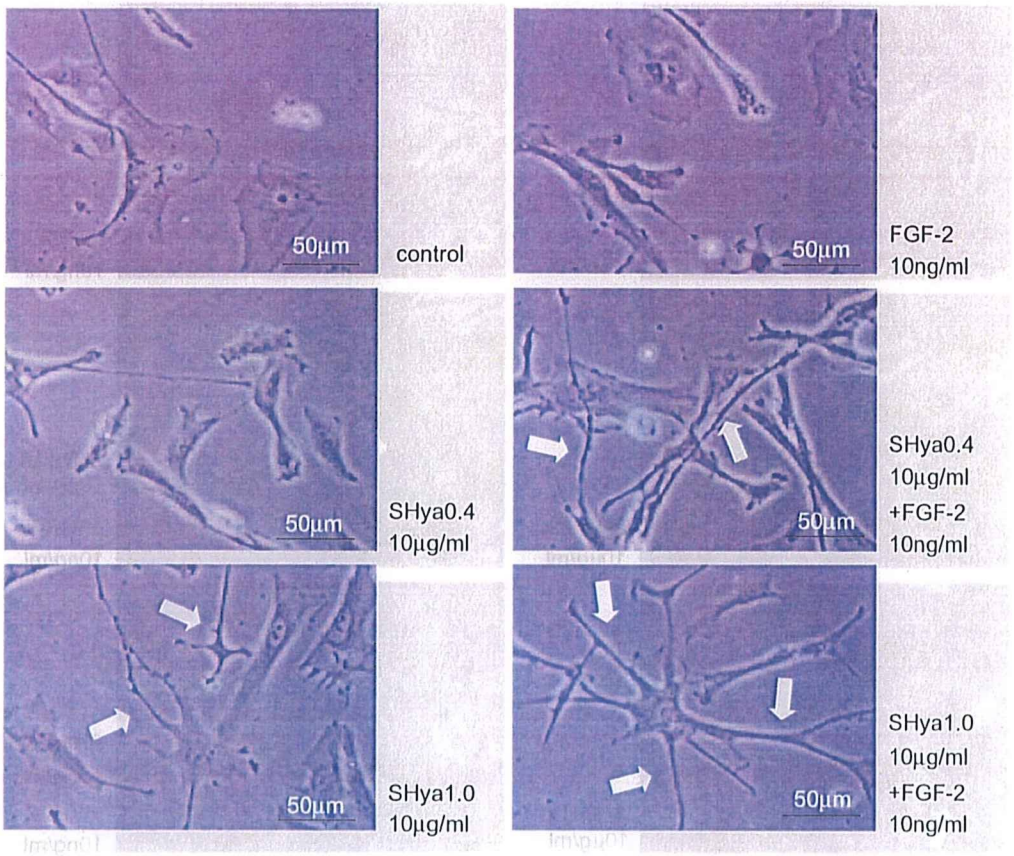

Fig. 6. Effects of SHya, FGF-2, and SHya with FGF-2 on morphological changes in cultured NHA over 10 days. The concentrations of each regent were 10 μg/ml SHya0.4, 10 μg/ml SHya1.0, and 10 ng/ml FGF-2. The arrow shows stellation of NHA.

compared with those at 5 days of culture (control). Treatment with SHya1.0 or SHya0.4 plus FGF-2 promoted NHA stellation, and treatment with SHya1.0 plus FGF-2 promoted NHA stellation dramatically (Fig. 6). After 20 days of culture, NHA cell bodies were enlarged markedly compared with 5 days of culture (control). Treatment with SHya0.4 plus FGF-2 transformed the NHA cell body into a thin cell body like a stella. Treatment with SHya1.0 partly promoted stellation but prevented cell body enlargement. Treatment with SHya1.0 plus FGF-2 promoted stellation markedly. Treatment with FGF-2 alone or FGF-2 with either SHya prevented cell body enlargement (Fig. 7). In 10 days of culture, treatment with SHya, FGF-2, or both increased GFAP and nestin mRNA expression and increased GFAP protein expression. These increases were markedly increased by the combination of either SHya plus FGF-2 (Fig. 8).

3.5. SLDT assay

The SLDT assay estimates cell–cell adhesion and gap-junctional intercellular communication by quantifying dye introduction into cells. Treatment with 10 μg/ml SHya1.0 increased dye influx into NHA significantly (Fig. 9).

3.6. Effect of SHya on mRNA expression

Treatment with SHya1.0 with or without FGF-2 increased FGF-2 and NGF mRNA expression. Treatment with SHya1.0 or FGF-2 increased midkine and IGF-1 mRNA expression. The combination of SHya1.0 and FGF-2 increased midkine mRNA expression remarkably. Treatment with 10 μg/ml SHya1.0 increased BDNF mRNA expression, but the addition of 10 ng/ml FGF-2 prevented BDNF mRNA expression due to SHya1.0 (Fig. 10).

4. Discussion

Current evidence indicates that astrocytes not only help maintain the physical structure of neurons but also contribute to formation of synapses and neural plasticity, in which astrocytes are transformed into active astrocytes (stellation) [5]. Therefore, astrocytes may be an effective target for therapy of central nervous system disorders and synaptic regeneration [23]. The purpose of this study was to elucidate the effects of SHya on astrocytes activities. We synthesized the sulfated hyaluronans SHya0.4 and SHya1.0, which have different numbers of sulfated groups, to estimate the quantitative effects of the number of sulfated groups introduced. First, we examined the biocompatibility and toxicity of SHyas on cultured cells. Nagahata et al. showed that 500 μg/ml synthesized SHya increased alkaline phosphatase activity in rat calvarial osteoblast [17]. A colony assay showed that SHya0.4 and SHya1.0 did not reduce V79 cell viability but treatment with 50 and 100 μg/ml SHya1.0 tended to reduce. MTT assay showed that SHya0.4 did not change mitochondrial activity but treatment with 100 μg/ml SHya1.0 reduced mitochondrial activity of NHA. Treatment with 10 μg/ml SHya1.0 tended to decrease mitochondrial activity, but the difference was not statistically significant. From these results, we suggested that 100 μg/ml SHya declined NHA proliferation and activity and we used 10 μg/ml SHya in the following study. Several glucosaminoglycans contribute to the activities of neurotrophic factors, and heparin, a sulfated glucosaminoglycan, binds FGF-2 and enhances FGF-2 bioactivity in the CNS

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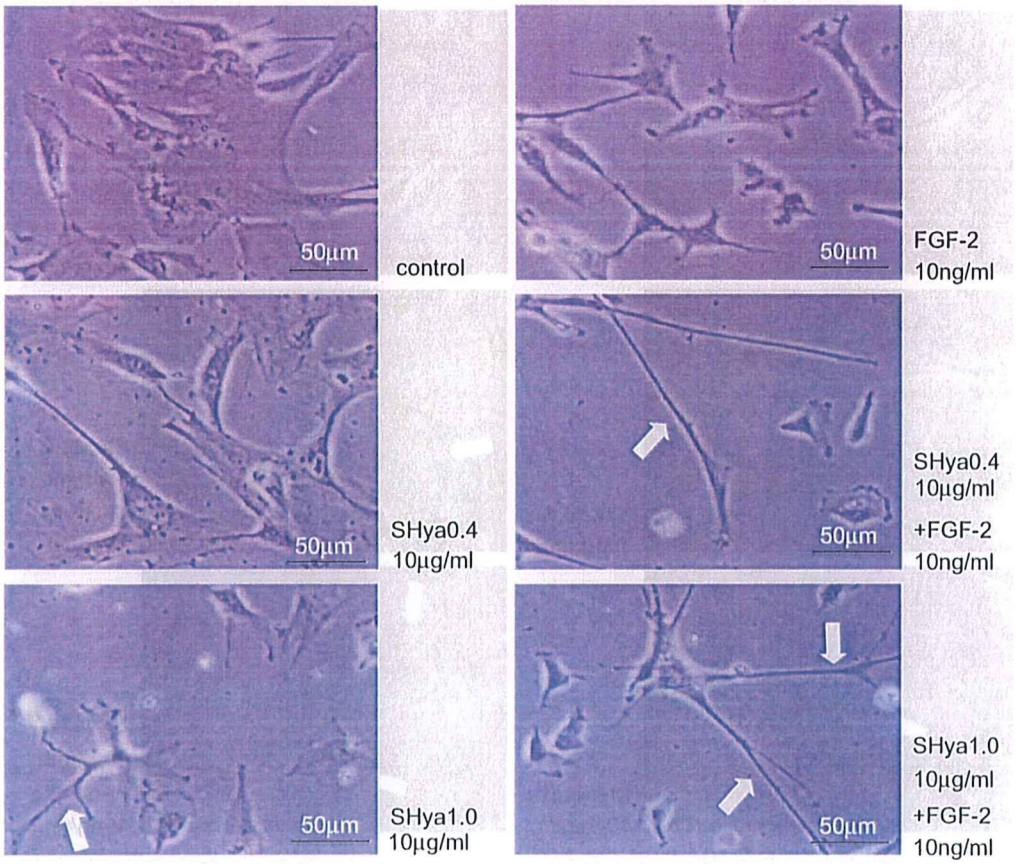

Fig. 7. Effects of SHya, FGF-2, and SHya with FGF-2 on morphological changes of cultured NHA over 20 days. The concentrations of each regent were 10 μg/ml SHya0.4, 10 μg/ml SHya1.0, and 10 ng/ml FGF-2. The arrow shows stellation of NHA.

[9,10]. Previous studies suggested that heparin enhances the effects of FGF-2 on NSC proliferation and neural network regeneration and that SHyas also enhanced FGF-2 activity and affected cell proliferation because SHya binds to FGF-2 [17,18]. Therefore, we examined the effects of 10 μg/ml SHya and SHya plus 10 ng/ml FGF-2 on astrocyte cultures. NHA proliferation increased smoothly until 30 days in in vitro culture, but cell proliferation virtually stopped after 30 days with NHA cell body enlargement (Figs. 4–7, control). The cell body enlargement stopped cell proliferation and caused cell senescence. Senescence in cells arrests cell growth in the G1 phase and changes the morphology and metabolism. Some of the senescence-associated changes that are common to many different cell types include cellular enlargement, increased lysosome biogenesis, and expression of a β-galactosidase that has a pH optimum of six [24]. Some reports suggested that 10 ng/ml FGF-2 increased cell proliferation of rat mesencephalic glia and human astrocytes [25,26]. In this study, treatment with 10 ng/ml FGF-2 promoted NHA proliferation and prevented cell enlargement (Figs. 4–7), and this result was consistent with previous reports. Other reports suggested that FGF-2 enhances growth and maintains the potential for multi-differentiation in human mesenchymal stem cells [27,28]. Old astrocytes have reduced neurotrophic effects compared to young astrocytes, and treatment of old astrocytes with FGF-2 recovered their neurotrophic effects on neurons [29]. These results suggest that treatment with FGF-2 maintained NHA cell proliferation and prevented NHA cell senescence.

Treatment with SHya0.4 or SHya1.0 did not affect NHA proliferation. On the other hand, the combination of one of the SHyas and FGF-2 increased NHA proliferation, although the increase was lower than after treatment of FGF-2 only (Fig. 4). We demonstrated that treatment with heparin had no effect on NHA cell proliferation and on FGF-2 activity in astrocyte proliferation (unpublished results). Sulfated groups are necessary to bind FGF-2 to sulfated glucosaminoglycans, and it was considered that the FGF-2 binding activity of sulfated glucosaminoglycans is proportional to the number of sulfated groups. However, the effects on NHA proliferation did not differ with the combination treatment of FGF-2 plus SHya0.4 or SHya1.0. These results suggested that the effects of SHya on astrocyte proliferation do not involve FGF-2 activity or reduce the FGF-2 activity in NHA proliferation.

The change of normal resting astrocytes into reactive astrocytes enhances neurotrophic factor production and synaptic plastic activities. Reactive astrocytes increase their levels of GFAP, an astrocyte marker protein, by themselves and transform the cell shape into a stella [7,30]. This cell transformation is promoted by phosphorylation of protein kinase K [7]. In this study, treatment with FGF-2 caused transformation of NHA. This is consistent with previously reported evidence that FGF-2 causes the stellation of astrocytes [30]. The use of SHyas for NHA culture promoted distinct stellation, and they were more effective in promoting NHA stellation than FGF-2. The combination of FGF-2 and SHya0.4 or SHya1.0 promoted distinct NHA stellation more than treatment of FGF-2 alone, and, in particular, FGF-2 and SHya1.0 caused marked stellation (Figs. 5–7). In addition, treatment with SHya1.0 increased mRNA expressions of GFAP and nestin, a neuron specific maker, and GFAP protein expression in astrocytes (Fig. 8). An increase in these protein expressions produced activation of astrocytes in some reports [1,7]. This result showed that NHA stellation was strongly

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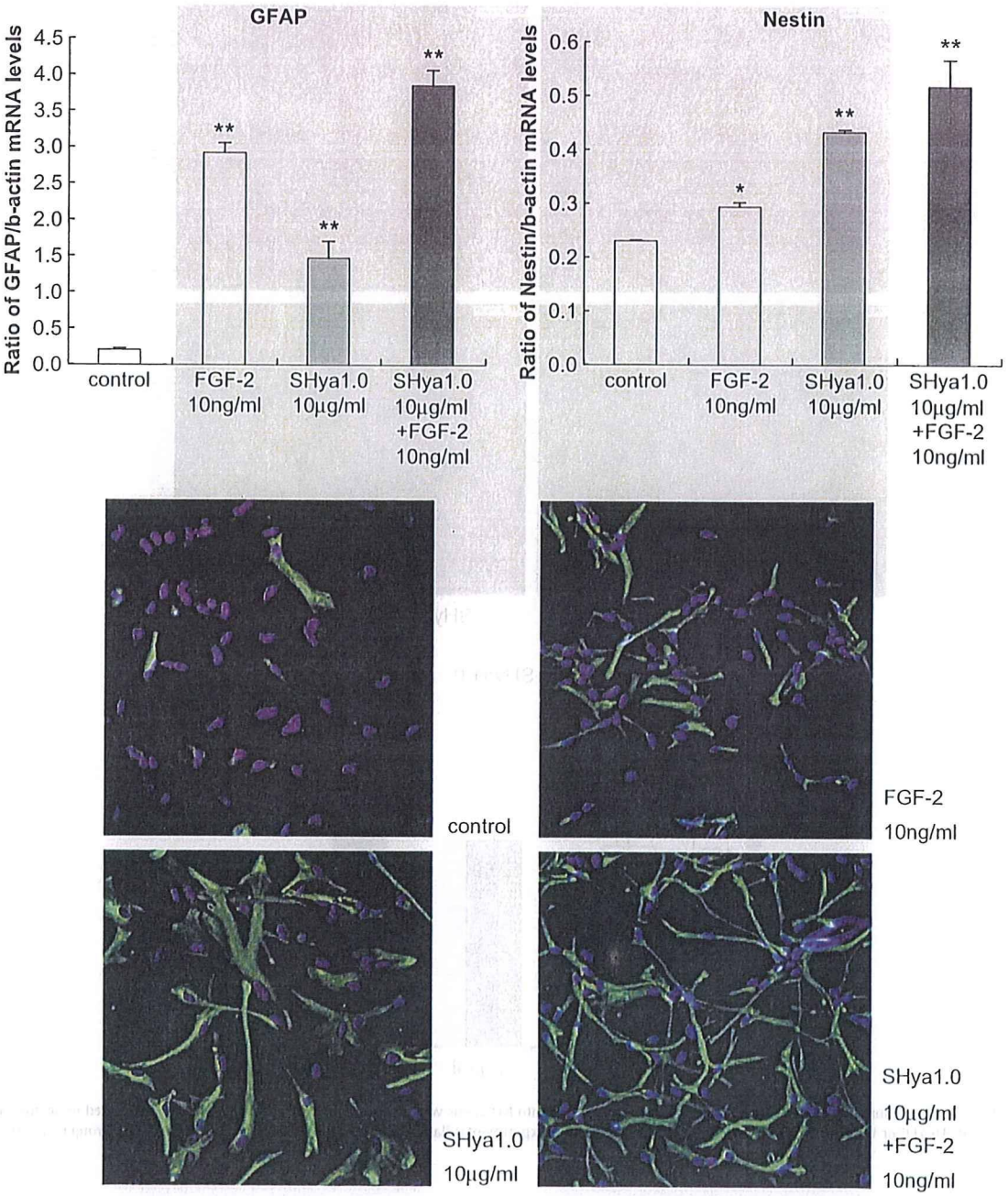

Fig. 8. Effect of SHya on GFAP and nestin mRNA expressions of astrocytes and immunostaining of GFAP. The concentrations of each regent were 10 μg/ml SHya1.0 and 10 ng/ml FGF-2. The arrow shows stellation of NHA. Values are mean ± SD ($n = 4$). Experimental data were significantly different from that of the control group (*, $p < 0.05$; **, $p < 0.01$).

enhanced by treatment with SHya1.0 and that FGF-2 with SHya transformed normal resting astrocytes to activating astrocytes depending on the D.S. of SHya but did not without FGF-2. Based on the assumption that transformation of NHA would increase connections between the NHA themselves, and we measured a cell–cell adhesion and gap-junctional intercellular communication. The SLDT assay showed that treatment with 10 μg/ml SHya1.0 increased the dye influx into NHA, indicating that gap junctions of astrocytes were increased and that cell–cell adhesions were intensified. It was previously shown that SHya increased the mRNA levels of the adhesion molecules *N*-cadherin and connexin43 in cultured rat calvarial osteoblasts [17]. We hypothesized that SHya increased a cell–cell adhesion by promoting NHA stellation and might increase adhesion molecules in NHA similar to that in calvarial osteoblast cells. Heparin, the average D.S. of which was 0.6, also promoted NHA stellation with FGF-2 [31]. The changes in NHA morphology via FGF-2 system depended on the D.S. of SHya, and the NHA transformations may have intensified the cells' crosstalk with neurons.

In the following study, we measured mRNA expressions of astrocyte-producing neurotrophic factors in NHA. Treatment with SHya1.0 increased mRNA expressions of FGF-2, midkine, BDNF, NGF, IGF-1 of astrocytes. These results also strongly suggest that SHya1.0 enhances astrocyte activity and supports neurocytes

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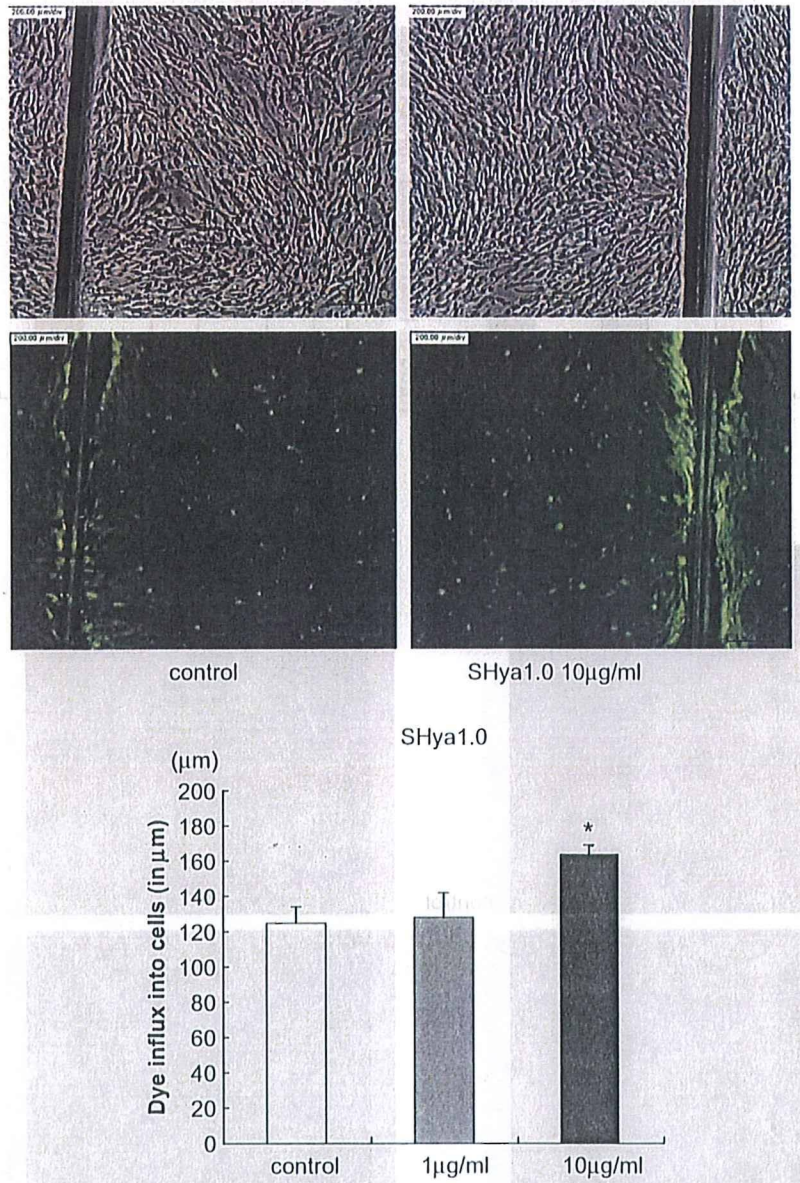

Fig. 9. Estimation of cell–cell adhesion of NHA by SLDT assay. The extent of dye influx into NHA cells was measured by the SLDT assay. NHA were cultured in normal NHA culture medium with 1, 10 μg/ml SHya1.0 or without (control). Values are mean ± SD ($n = 4$). Experimental data were significantly different from the control group (*, $p < 0.05$).

functionally and structurally. In particular, FGF-2 performs some functions of neurocytes, and together with EGF, which is needed for survival of neural stem cells. Glial cells cultured in the presence of FGF-2 and EGF expressed nestin in the culture dish [6,28,29]. These reports show that these trophic factors closely participate in proliferation and differentiation of neural stem cells and neurons. In addition, FGF-2 and midkine bind heparan sulfates and enhance neurotrophic activity by themselves. A combination of FGF-2 and heparin promoted astrocyte stellation [31]. Midkine and BDNF are also involved in neuronal growth, and interaction of these neurotrophic factors with chondroitin sulfate caused the expansion of dendrites [11]. That is, the effects of sulfated glucosaminoglycans on the CNS involve heparin-binding neurotrophic factors such as FGF-2, midkine, and others. Our study showed that the FGF-2, midkine, and BDNF levels were increased significantly by SHya treatment. FGF-2 bound to SHya and activated itself [17,18]. Midkine also binds to heparan sulfates and chondroitin sulfates, like FGF-2, and we hypothesized that SHya interacts with midkine [30,31]. Another report indicated that direct interaction of chondroitin sulfate oligosaccharides, which have installed sulfate groups, with midkine and BDNF promoted neuronal growth in cultured hippocampal neurons [11]. Thus, FGF-2, midkine, and BDNF are involved in the stellation of astrocytes by SHya. On the other hand, some neurotrophic factors, such as NGF, FGFs, and BDNF, are secreted for neurogenesis and neuroprotection in the injured brain. IGF-1 is also involved in neuroprotection, neurogenesis, and glucose utilization [32,33]. A recent report showed that IGF-1 contributed to the maintenance of youthful levels of cognition during aging in mammals [34]. These results suggested that IGF-1 might prevent a decline in the activity of neurocytes in the CNS. These trophic factors work together or independently, and their expressions may be mutually regulated [35–37]. In this study, treatment with FGF-2 increased the midkine mRNA level, but it did not change the BDNF and NGF mRNA levels. Apparently, SHya itself

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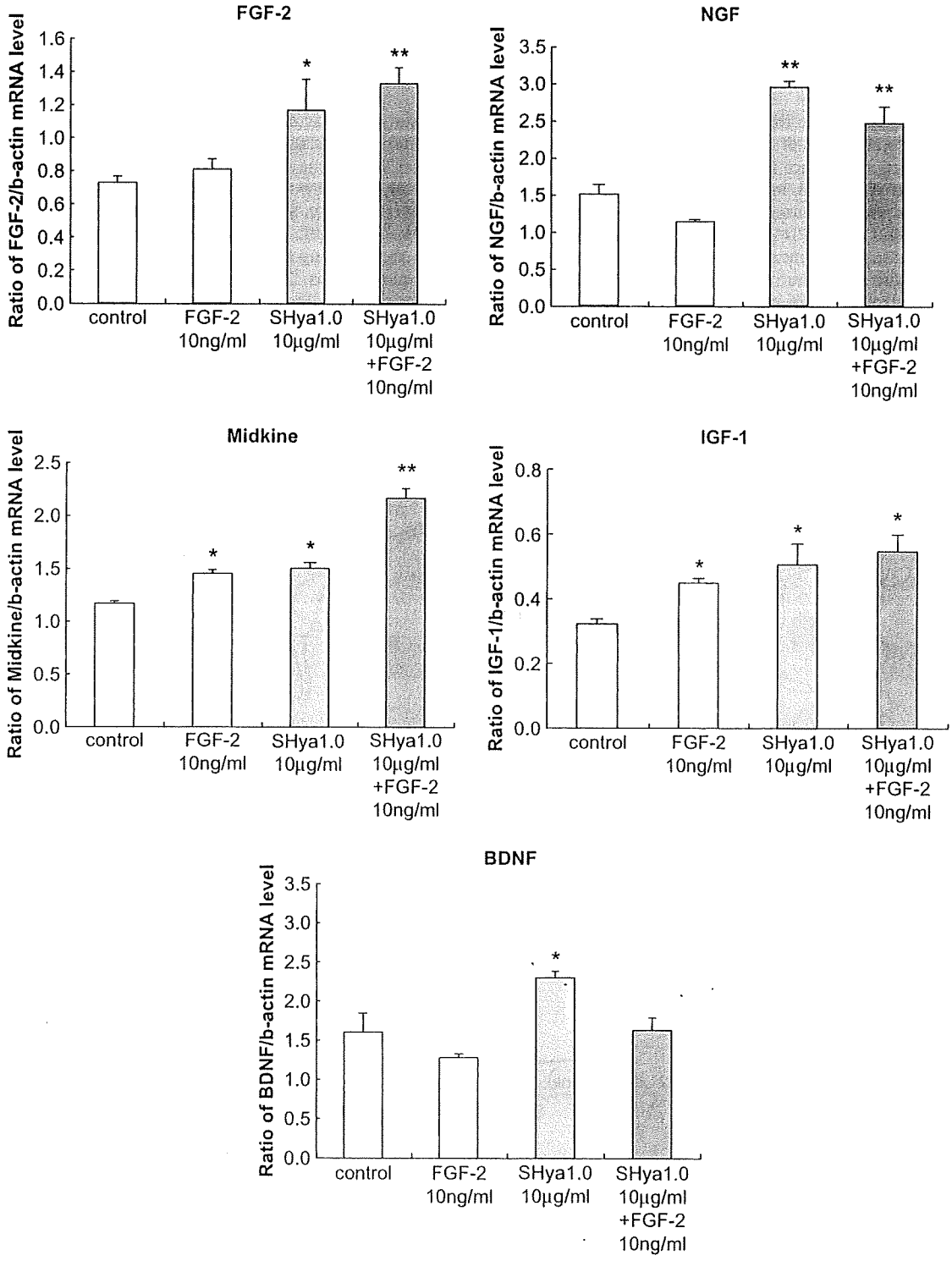

Fig. 10. Effects of SHya on mRNA expressions of FGF-2, midkine, and BDNF in astrocytes. NHA were cultured in normal NHA culture medium (control) or with 10 μg/ml SHya, 10 ng/ml FGF-2, or SHya with FGF-2. Values are mean ± SD ($n = 3$). Experimental data were significantly different from the control group (*, $p < 0.05$; **, $p < 0.01$).

and activation of these neurotrophic factors by SHya promoted the mRNA expression of these factors.

Heparin has a similar effect to that of SHya, and heparin transformed normal astrocytes to the stellar morphology [30]. Neurocan, a brain specific chondroitin sulfate, interacted with FGF-2 and caused the multiplication of neural stem cells in the neonatal rat brain [38]. Chondroitinase ABC, a digestive enzyme of chondroitin sulfate proteoglycan, enhanced outgrowth-associated protein-43-positive fibers after rat spinal cord injury [39]. Midkine, a heparin-binding growth factor, also promoted neural precursor

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cell growth [31]. These reports suggest that sulfated glucosaminoglycans enhanced the activity of the heparin-binding growth factor midkine and FGF-2. However, we hypothesized that the effects of SHya on astrocytes were not limited to enhancement of neurotrophic factor activities. In our study, the effect of 20 ng/ml FGF-2 on stellation did not differ from that of 10 ng/ml FGF-2 (data not shown), but a combination of 10 ng/ml FGF-2 and SHya0.4 or SHya1.0 promoted more distinct NHA stellation than treatment with 10 or 20 ng/ml FGF-2, and, in particular, the combination of 10 ng/ml FGF-2 and SHya1.0 caused marked stellation (Figs. 4–6). In addition, BDNF and NGF mRNA levels also were increased by SHya, but FGF-2 reduced this increase. Therefore, SHya not only activates neurotrophic factors but also affects NHA stellation and enhances the neurotrophic factor-producing activity of astrocytes. That is, SHya is an efficient inducer of activation of astrocytes (Fig. 11).

5. Conclusion

This study demonstrated the effects of sulfated hyaluronan on proliferation, morphological transformation, and mRNA expression of neurotrophic factors in astrocytes. SHya promoted the stellation and neurotrophic factor-producing activities of astrocytes. The combination of SHya and FGF-2 promoted stellation of astrocytes more than SHya alone and increased astrocyte proliferation. The effect of SHya on astrocytes depended on the D.S. of SHya and involved FGF-2 activity. SHya alone promoted astrocytes stellation and increased cell–cell adhesion. These results suggest that SHya supports restructuring of the network between astrocytes or astrocytes and neurons. SHya increased FGF-2, midkine, and BDNF mRNA expressions. These neurotrophic factors are involved in neurogenesis, neuroprotection, and neural dendrite expansion. These neurotrophic factors interact with SHya functions and

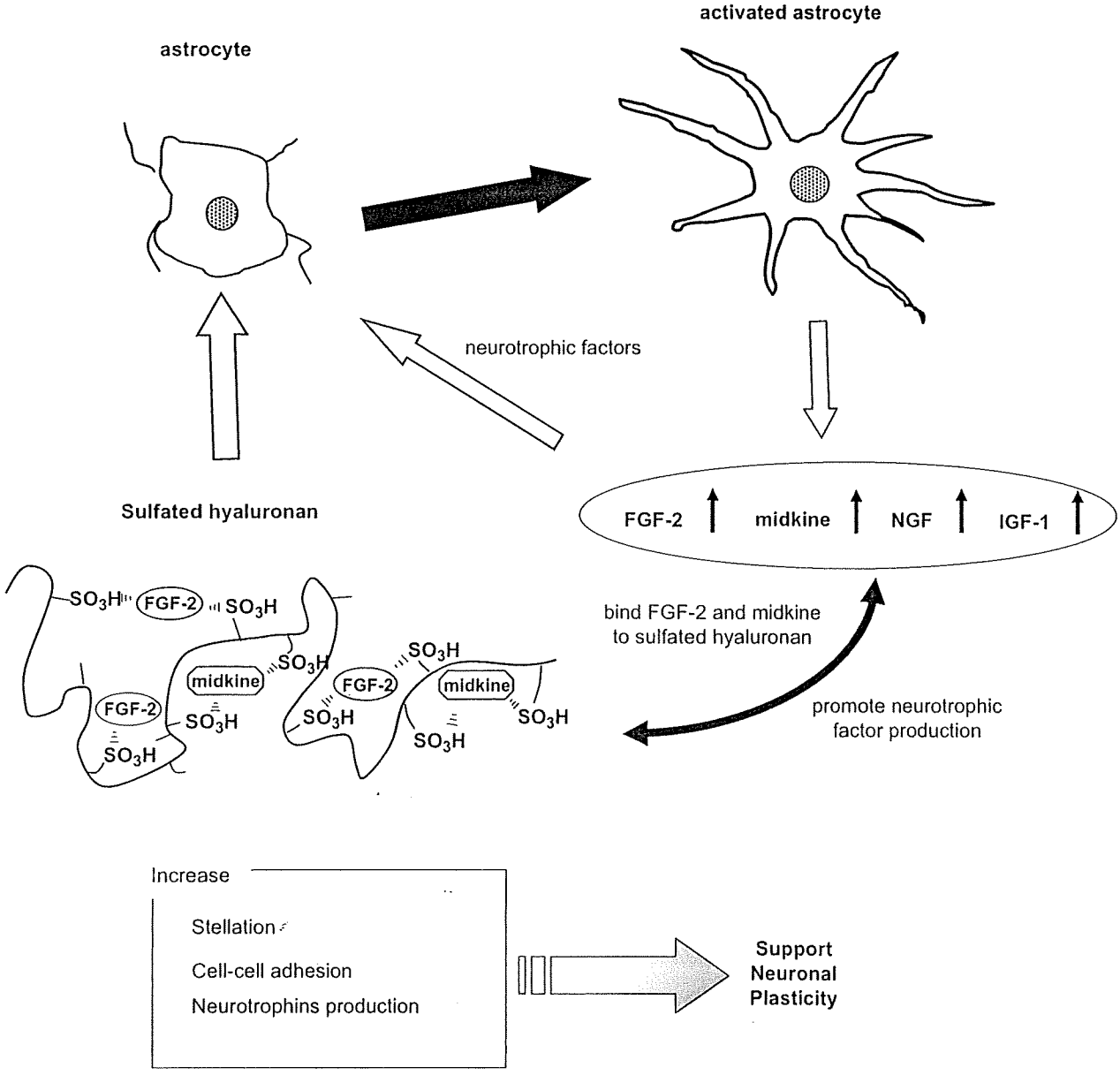

Fig. 11. Proposed mechanism of the effect of SHya on human astrocyte activities.

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involve astrocyte stellation. Finally, astrocyte activities are required for the survival of neurons and regeneration of the neural network after brain injury, surgery, and neural stem cell transplantation. Synthesized SHya may enhance the survival of neurons and neural stem cells and affect neural plasticity by activating astrocytes. We expect that SHya will be applicable to safe and reliable medical biomaterials for neuroprotection and neurogenesis.

Acknowledgments

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ORIGINAL ARTICLE

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Toshie Tsuchiya, PhD

Safety evaluation of surgical materials by cytotoxicity testing

Abstract The cytotoxicity of three kinds of commercially available absorbable hemostats [oxidized cellulose (Surgicel, gauze and cotton types), microfibrillar collagen (Avitene), and cotton-type collagen (Integran)] and one adhesion barrier [sodium hyaluronate and carboxymethylcellulose membrane (Seprafilm)] were comparatively assessed by a colony assay using V79 cells and a minimum essential medium (MEM) elution assay in combination with a neutral red assay using L929 cells. Strong cytotoxicity was detected for Surgicel by both the MEM elution assay and the colony assay. For Avitene, both methods revealed weak cytotoxicity. For Seprafilm, no cytotoxicity was detected by the MEM elution assay, while a moderate degree of cytotoxicity was observed in the colony assay. For Integran cytotoxicity was not detected by either the MEM elution or the colony assay. The results of the different methods showed some inconsistency in terms of the degree of cytotoxicity of the materials. It is proposed that the combination of two or more sensitive cytotoxicity testing methods for the evaluation of biomaterials is necessary to avoid false-negative results for biomaterials at the preclinical stage. Furthermore, investigation of the correlation between the cytotoxicity and the extraction period of the surgical materials is helpful for predicting the effect of prolonged in vivo use of biomaterials on surrounding cells, tissues, and organs.

Key words Safety evaluation · Surgical materials · MEM elution assay · Colony assay

Introduction

Many types of biomaterials have been utilized in surgical techniques and tissue engineering. Assessment of the cytotoxicity based on several sensitive and quantitative cytotoxicity testing methods is a necessary step in the evaluation of biocompatibility of all biomaterials. In particular, it is an important step in the safety evaluation of implants, absorbable biomaterials, and biomedical devices. At present, although a number of cytotoxicity testing methods have been developed and safety evaluations are being standardized and described by various national and international standardization institutes,[1,2] there are still many reports of problems caused by clinically utilized biomaterials.[3–9] To avoid false-negative results, the safety of biomaterials should be sufficiently assessed at the preclinical stage.

In the present study, we comparatively assessed the cytotoxicity of three kinds of commercially available absorbable hemostats [oxidized regeneration cellulose (Surgicel, gauze and cotton types), microfibrillar collagen (Avitene), and cotton-type collagen (Integran)] and an adhesion barrier [sodium hyaluronate and carboxymethylcellulose membrane (Seprafilm)] by two different cytotoxicity testing methods. These surgical materials have been widely utilized in clinical applications; however, in reports by the US Food and Drug Administration of clinical problems caused by biomedical devices, Seprafilm, Surgicel, and Avitene were reported to have been implicated in 226, 44, and 24 events, respectively, in the period from January 1, 1996, to August 30, 2006.[10] It was reported that inhibition of bone regeneration, foreign-body reaction, inflammation reaction, and abscess formation were caused by using these materials in the clinical setting. Although the frequency of significant adverse reactions was not clear, wound infection, abscessus, peritonitis, and sepsis have also been reported.[10]

The MEM elution assay is commonly used for qualitative evaluation of polymeric materials and has been widely used as a standardized safety evaluation method in various nations. To quantitatively assess the cytotoxicity of the sample materials, the neutral red (NR) assay was combined with the MEM elution assay. The colony assay cytotoxicity testing method developed by Tsuchiya et al. is recognized as a sensitive, quantitative, and reproducible cytotoxicity testing method for medical devices.[1] The main feature of the colony assay is the use of a few cells in the test, testing

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the effect of biomaterials on the colony formation ability of the cells, and the testing period is 1 week. This method can obtain results similar to those of in vivo testing and can also predict the effect of prolonged in vivo use of biomaterials.[11] In contrast, the MEM elution assay in combination with the NR assay uses a great number of cells to test the effect of biomaterials on the survivability of cells, and the testing period is 24 h.

The cytotoxicities of the three hemostats and one adhesion barrier were assessed using these different testing methods, and the experimental results will be discussed comparatively. The main purpose of present study was not only detection of the cytotoxicity of each sample, but also investigation of the correlation between the cytotoxicity of biomaterials and clinical problems, and finding a definitive cytotoxicity testing methodology for biomaterials at the preclinical stage.

Furthermore, to predict the prolonged in vivo effect of these surgical materials on surrounding cells, tissues, or organs, the relationship between the degree of cytotoxicity and the extraction period used to prepare the sample for the assay was evaluated by the colony assay cytotoxicity testing method using V79 cells. The experimental results were compared with those of the standard reference materials evaluated by the same procedure.

Materials and methods

Three kinds of locally absorbable hemostats [oxidized regeneration cellulose (Surgicel, gauze and cotton types), microfibrillar collagen (Avitene), and cotton-type collagen (Integran)] and one type of adhesion barrier [sodium hyaluronate and carboxymethylcellulose membrane (Seprafilm)] were tested in this study. In addition, two kinds of segmented polyurethane films containing 0.1% zinc diethyldithiocarbamate (SRM-A) and 0.25% zinc dibutyldithiocarbamate (SRM-B) were used as positive standard reference materials, and a high-density polyethylene sheet (SRM-C) (thickness about 0.5 mm) was used as the negative standard reference materials for colony assay. All standard reference materials were kindly provided by Hatano Research Institute, Food and Drug Safety Center (Kanagawa, Japan).

Sample preparation

The product names, and ingredients of the tested materials are summarized in Table 1. Several pieces of the sterile sample were placed in a sterilized tube and culture medium was added at a ratio of sample/medium of 6 cm^2/ml.[10] Eagle's MEM supplemented with 10% fetal calf serum (FCS) (10% FCS-MEM) (MEM, Gibco, Grand Island, NY, USA) was used for the MEM elution assay in combination with the NR assay, and Eagle's MEM supplemented with 5% FCS, nonessential amino acids, and 1 mmol/l sodium pyruvate (5% FCS-GMNP) was used for the colony assay method. After incubation at 37°C in a saturated humidified atmosphere of 5% CO_2 and 95% air for 1 day, the extract solution designated as 100% extract was separated by centrifugation and decantation. The pH of the extract medium solution was measured and the detailed results of all samples are summarized in Table 1. The 100% extract was serially diluted with the corresponding medium to give 50%, 25%, 12.5%, and 6.25% extract solutions. Extracts showing acidity were neutralized with 1 M NaOH aqueous solution to a pH value of 7.4 and the neutralized extracts were also used in the assays.

Cytotoxicity test

MEM elution assay in combination with NR assay

In this study, the MEM elution was performed according to USP XXII(87) *Biological Reactivity Tests, in Vitro*. Murine fibroblast L929 cells were obtained from the Japanese Health Science Research Resources Bank (Osaka, Japan) and were grown in 10% FCS-MEM medium supplemented with nonessential amino acids at 37°C in a saturated humidified atmosphere of 5% CO_2 and 95% air.

L929 cells (5×10^4) in 0.5 ml of 10% FCS-MEM were seeded in each well of a 24-well plate and incubated at 37°C in the saturated humidified atmosphere for 24 h. The medium was replaced with or without serially diluted extract. The cells were observed microscopically after 24-h exposure to the extract.

NR is a vital dye that is actively endocytosed within lysosomes of viable cells and provides an index of cell viability. After the MEM elution assay, the cytotoxicity of the

Table 1. Biomaterials utilized to assess cytotoxicity

Sample	Form	Material	Extraction conditions		
			Surface area/medium (cm^2/ml)	Weight/medium (g/ml)	pH
Surgicel	Gauze type	Oxidized cellulose	6	0.021	2.9 ± 0.1
	Cotton type		6	0.079	2.8 ± 0.1
Avitene	Sheet	Microfibrillar collagen	6	0.09	4.2 ± 0.1
Integran	Sheet	Cotton type collagen	6	0.012	8.4 ± 0.1
Seprafilm	Sheet	Sodium hyaluronate and carboxymethylcellulose	6	0.029	6.8 ± 0.1
SRM-A	Sheet	Polyurethane (0.1% ZDEC)	6	0.12	7.2 ± 0.1
SRM-B	Sheet	Polyurethane (0.5% ZDBC)	6	0.12	7.2 ± 0.1
SRM-C	Sheet	Polyethylene	6	0.12	7.2 ± 0.1

SRM, standard reference material; ZDEC, zinc diethyldithiocarbamate; ZDBC, zinc dibutyldithiocarbamate

serially diluted extract on the L929 cells was quantitatively assessed by measuring uptake and accumulation of NR by the viable cells.

NR was dissolved in 10% FCS-MEM at a concentration of 50 μg/ml and the fine dye crystals were removed by centrifugation at 2000 ×g for 10 min. After observation of the MEM elution assay, the medium was replaced by 0.5 ml of medium containing NR and incubation was continued for 3 h at 37°C. After washing away the excess NR solution using phosphate buffered saline (PBS) (–) three times, the cells were fixed with 0.5 ml of 1% formalin and 1% $CaCl_2$ (v/v) and the dye was extracted by 0.5 ml/well of 1% acetic acid in 50% (v/v) ethanol solution. Absorbance of the obtained extract solution was recorded at 540 nm using a μQuant microplate reader (Bio-tek, Winooski, VT, USA).

Colony assay

The colony assay using V79 cells followed the *Japanese Guidelines for Basic Biological Tests of Medical Materials and Devices-Part III: Cytotoxicity Test.* Chinese hamster fibroblast V79 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and were grown in Eagle's MEM supplemented with 10% FCS at 37°C in a saturated humidified atmosphere of 5% CO_2 and 95% air. Several pieces of the sterile samples were placed in a sterilized tube and Eagle's MEM supplemented with 5% FCS, nonessential amino acids, and 1 mmol/l sodium pyruvate (5% FCS-GMNP) was added so that the sample/medium ratio was 6 cm^2/ml. After incubation at 37°C in the humidified atmosphere for 1, 7, and 14 days, the extract, designated 100% extract, was separated by centrifugation and decantation.

A cell suspension (0.5 ml) with a concentration of 100 cell/ml was placed in each well of a 24-well plate and the plate was incubated for 4 h. Then, the medium was replaced with 0.5 ml of the serially diluted extract solution or the medium without extract (as a control), and the cells were cultured for 7 days. After the 7-day culture, the cells were fixed with methanol and stained with 5% Giemsa staining solution. The number of cell colonies in each well was counted, and the relative colony forming rate was calculated as the ratio of the number of cell colonies in the sample to that in the control. The cytotoxicity of the extracts was quantitatively expressed as IC_{50} (%), which is the extract concentration corresponding to the colony forming rate of 50%.

Data presentation and statistical analysis

The viability index for the NR method was calculated as follows: % sample viability = (A/B) × 100, where A is the optical density of cells cultured with an extract (sample) and B is the optical density of cells cultured without the extract (as control). All values were obtained from three or four sets of the same experiments and expressed as mean values ± SD. Differences among the groups were evaluated using analysis of variance (ANOVA), and $P < 0.05$ was considered statistically significant.

Results

Figure 1 shows results of the MEM elution assay with and without (as a control) the extract from Surgicel (gauze type, Surgicel 1) (Fig. 1A,B) and quantitative assessment of Surgicel 1 by the MEM elution assay combined with the NR assay (Fig. 1C–J). As shown in Fig. 1A, more than 50% of the cells in the 100% extract of Surgicel 1 had a round shape. However, after staining with NR (Fig. 1C), all the cells were confirmed not to be viable, indicating that the MEM elution assay alone is not adequately sensitive and is unsatisfactory for the evaluation of biomaterials. In the control (Fig. 1D), all the cells were clearly stained with NR. For a 50% extract of Surgicel 1 (Fig. 1E), less than 20% of the cells were visible compared to the control, but almost all of the existing cells took up and accumulated the viability dye, NR. Overall, Fig. 1 clearly shows that the viable cell ratio increases with a decrease in the concentration of the extract both before and after neutralization. After neutralization, the viable cell ratio was higher compared to that for the same concentration of the extract solution before neutralization.

Figure 2 shows the relative colony forming rate of V79 cells cultured with various concentrations of extract solution of Surgicel 1 in the colony assay. The colony forming rate decreased with increasing extract concentration, and the colony formation of V79 cells was not observed when the concentration of the extract was greater than 25%. The calculated IC_{50} value for Surgicel 1 was very low at an extract concentration of about 16.5%. Moreover, the cytotoxicity of Surgicel 1 extract solution was slightly reduced after neutralization, but the IC_{50} was still very low at 18.5%.

The cytotoxicity of cotton-type Surgicel (Surgicel 2) was also evaluated by the same testing methods. Figure 3 shows the results of the MEM elution assay combined with the NR assay for various concentrations of the Surgicel 2 extract. More than 50% and 70% of cells were round in the 100% extract and 50% extract solutions, respectively. After staining with NR, as clearly shown in Fig. 3A–F, only cells in extract solutions concentrations of less than 25% endocytosed and accumulated the NR dye. Figure 3G,H shows the corresponding cell viability indices (% of control) of the various concentrations of extract solution of Surgicel 2 before and after neutralization. To compare the morphology of dead and viable cells, Giemsa staining was performed after the NR assay. It was clearly shown that the dead cells had a much more angular shape than the viable cells.

Figure 4 shows the results of the colony assay with various concentrations of the extract solution from Surgicel 2 before and after neutralization. The colony formation of V79 cells was not observed in extract solutions with concentrations above 25% and 50% before and after neutralization, respectively. The IC_{50} of the extract before neutralization

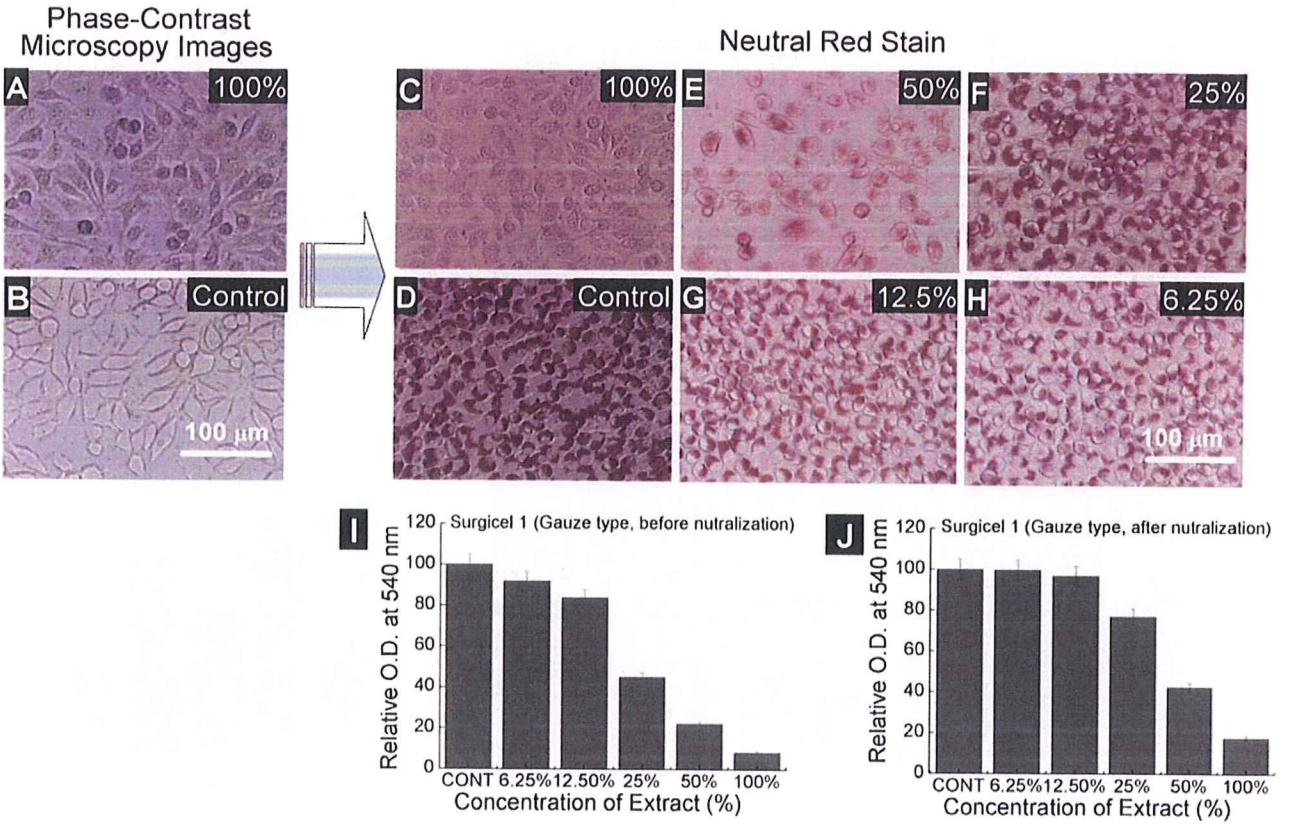
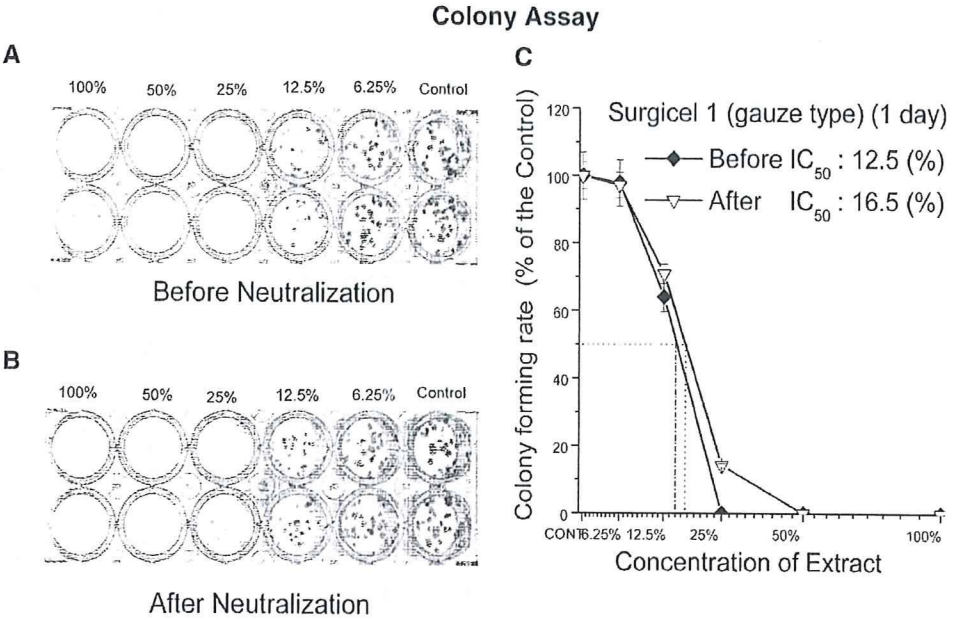


Fig. 1. Observation of L929 cell morphology on culture with extracted medium of Surgicel 1 (gauze type) and after staining with neutral red (**A–H**), and the cell viability of L929 cells before (**I**) and after neutralization (**J**). **A**, 100% extract solution tested by MEM elution assay; **B**, control (without any extract solution); **C**, neutral red staining of 100% of extract solution; **D**, neutral red staining of control; **E**, 50% extract solution; **F**, 25% extract solution; **G**, 12.5% extract solution; **H**, 6.25% extract solution. *O.D.*, optical density

Fig. 2. Colony formation of V79 cells cultured with extract medium of Surgicel 1: **A** before and **B** after neutralization. **C** The plating efficiencies of V79 cells cultured with the extract of Surgicel 1 (before and after neutralization)



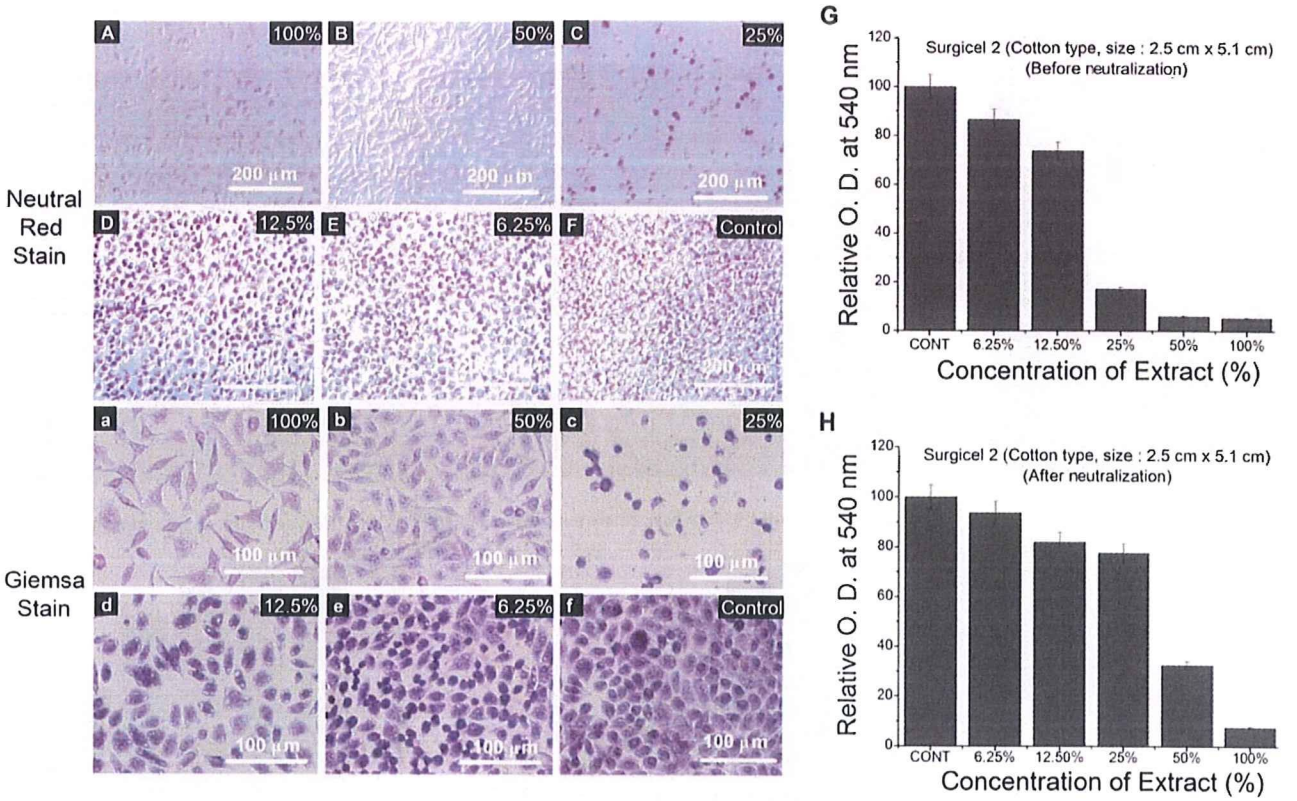
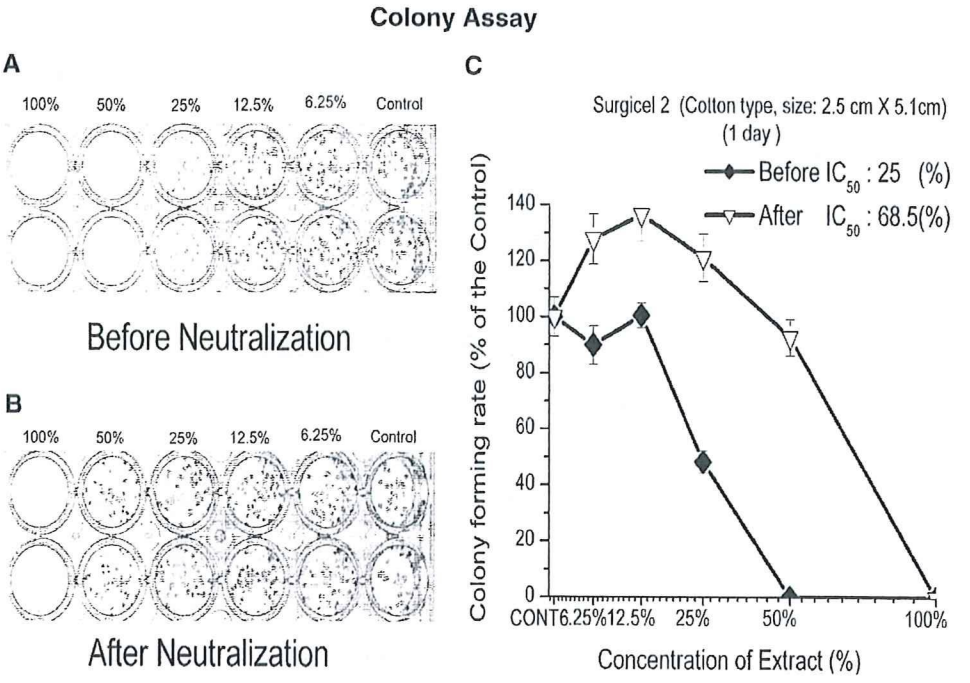


Fig. 3. Observation of L929 cell morphology on culture with extract medium of Surgicel 2 (cotton type) stained by neutral red (**A–F**) or Giemsa solution (**a–f**) and the cell viability of L929 cells before (**G**) and after neutralization (**H**). **A** and **a**, 100% extract solution; **B** and **b**, 50% extract solution; **C** and **c**, 25% extract solution; **D** and **d**, 12.5% extract solution; **E** and **e**, 6.25% extract solution; **F** and **f**, control (without extract solution)

Fig. 4. Colony formation of V79 cells cultured with extract medium of Surgicel 2: **A** before and **B** after neutralization. **C** The plating efficiencies of V79 cells cultured with the extract of Surgicel 2 (before and after neutralization)



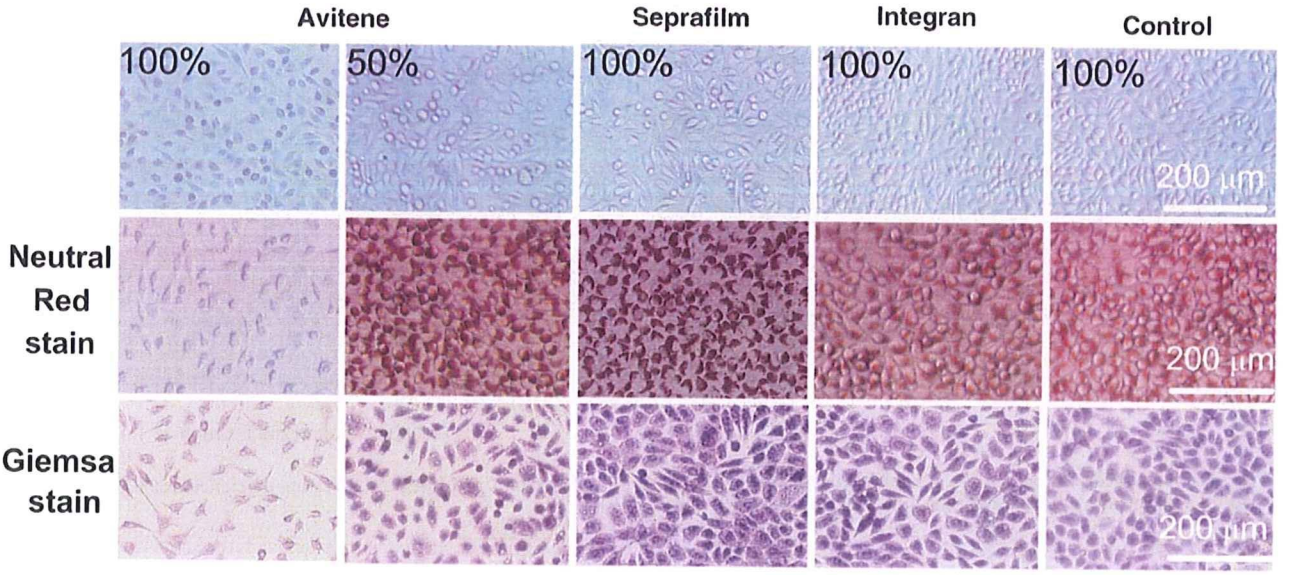


Fig. 5. Observation of L929 cell morphology of samples and control after staining with neutral red and Giemsa solution

was calculated to be 22.5%; however, after neutralization, it dramatically increased to the much higher value of 68.5%, indicating that the cytotoxicity of Surgicel 2 is strongly related to the acidic condition. In the extract solutions both before and after neutralization, the colony size decreased with increasing extract concentration.

The results of the MEM elution assay, NR assay, and Giemsa staining for 50% and 100% extract solutions of Avitene and 100% extract solutions of Seprafilm, Integran, and the control are shown in Fig. 5. Except for cells in the 100% extract solution of Avitene, all the rounded cells took up and accumulated NR dye. There was no significant difference between the viability of L929 cells cultured with medium extracts of Seprafilm, Integran, and the control, indicating that cytotoxicity was not detected for Seprafilm or Integran in the MEM elution assay. For Avitene, the viability of L929 cells cultured with a 50% extract solution also showed no significant difference with that of the L929 cells cultured with the control. The L929 cell viability cultured with various concentrations of extract solutions of Avitene, Seprafilm, and Integran are plotted in Fig. 6. The viability indices of L929 cells in the extract solutions of Avitene decreased steeply with increases in extract concentration when the extract concentration was above 50%, while no significant variation was observed for that of the L929 cells in the extract solutions of Seprafilm and Integran.

Figure 7 shows the results of the colony assay with various concentrations of extract solutions of Avitene, Seprafilm, and Integran. Moderate cytotoxicity was observed in the extract solutions of Separafilm at concentrations above 25%, and a weak cytotoxicity was detected in the extract solutions of Avitene at concentrations above 50%. For Integran, the colony forming rate decreased gradually with increasing extract concentration, and the relatively high colony forming rate of 37.4% was obtained even at

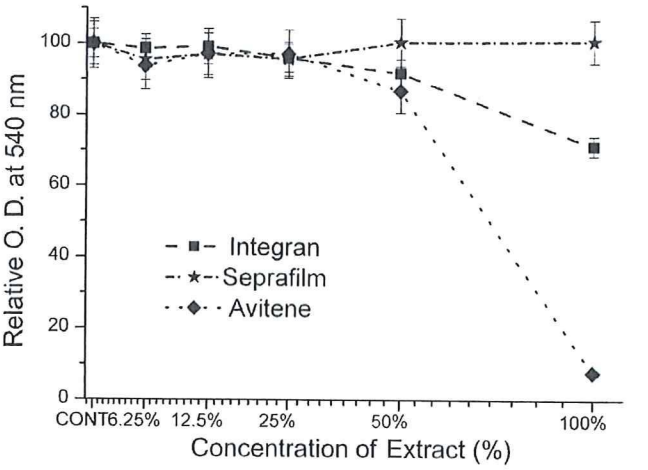


Fig. 6. Cell viability of L929 cells cultured with the extracts of Avitene, Seprafilm, and Integran

100% extract concentration. The IC_{50} of the extract solutions were calculated to be 72.5% for Avitene, 31.5% for Seprafilm, and 87.5% for Integran.

The results of the MEM elution assay and the colony assay for the 100% extract solutions of the materials are summarized and compared in the Table 2. Based on our results, the cytotoxicity of all tested materials was grade 2 or zero in the MEM elution assay. However, the NR assay indicated that only a few cells survived in the extract from Surgicel and Avitene. Furthermore, in the colony assay, colony formation (nearly 40%) was observed only in the 100% extract solution of Integran.

In this study, we also carried out cytotoxicity assessments of extract solutions of these biomaterials extracted for different periods by using the colony assay. The relationship between the cytotoxicities of the samples and the extraction

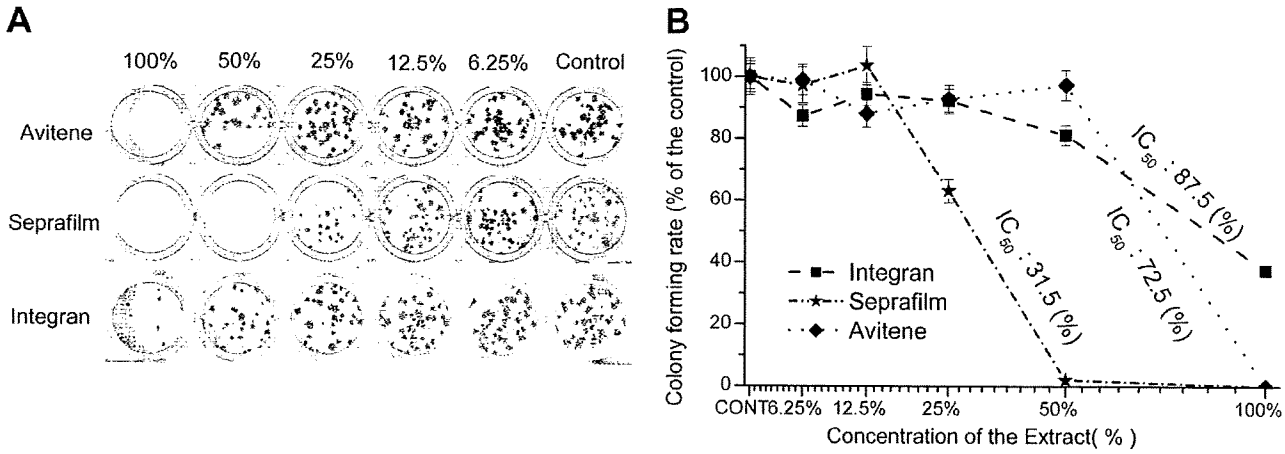


Fig. 7. **A** Colony formation of V79 cells cultured with the extracts of Avitene, Seprafilm, and Integran. **B** Plating efficiencies of V79 cells cultured with the extracts of Avitene, Seprafilm, and Integran

Table 2. Results of the MEM elution and the colony assays for 100% extracts of various surgical materials and the relationship between their cytotoxicities and extraction period

Sample	100% Extract (1 day)			Colony assay IC_{50} (%)			Adverse effects
	MEM elution assay		Colony assay	1 day	7 days	14 days	
	Grade of MEM elution assay	Relative O.D. at 540 nm (NR)	Colony forming rate (%)				
Surgicel gauze	2 (pass)	5.12 (3)[a] (fail)	0	14.5	9.5	9.0	Inhibited bone regeneration
Surgicel cotton	2 (pass)	5.36 (3)[a] (fail)	0	26.5	–	–	
Avitene	2 (pass)	7.74 (3)[a] (fail)	0	72.5	42.5	35	Abscess formation
Integran	0	71.4 (2)[a] (pass)	37.37	78.5	–	–	
Seprafilm	0	100	0	31.5	29	28	Foreign body reaction,
SRM-A	–	–	0	1.45	1.55	4.1	inflammation reaction, and
SRM-B	–	–	0	36.5	34	33	abscess formation
SRM-C	–	–	85.93	–	–	–	
ZDEC	–	–	0	0.25 (μg/ml)	0.75 (μg/ml)	>4 (μg/ml)	
Control	0	100	100	100	100	100	

MEM, minimum essential medium; O.D., optical density; NR, neutral red; IC_{50}, extract concentration corresponding to a colony forming rate of 50%; –, not tested

[a] Numbers correspond to the grade of MEM elution assay by the NR assay method

period is summarized in Table 2. The IC_{50} values obtained by the colony assay slowly decreased with increases in the extraction period up to 7 days for Surgicel 1, Avitene, and Seprafilm, indicating that the cytotoxicities of these materials increase with increases in the extraction period. Not much change was observed for the IC_{50} values of SRM-A and SRM-B.

Discussion

In this study, the cytotoxicities of four kinds of widely utilized biomaterials were tested by the MEM elution assay in combination with the NR assay and a colony assay. The MEM elution assay in combination with the NR assay assesses the cytotoxicity of biomaterials through testing the survivability of a great number of cells in the extract solutions of biomaterials. The colony assay assesses the cytotoxicity of biomaterials by testing the proliferation ability of a few cells in the extract solutions of biomaterials. The number of tested cells, cell behavior, and the testing periods are different between these cytotoxicity testing methods, so that they reveal different cytotoxic effects of biomaterials.

For Surgicel 1 and Surgicel 2, both methods revealed strong cytotoxicity, indicating that these absorbable hemostat materials not only affect the survivability, but also affect the proliferation ability of surrounding cells. For Avitene, both methods revealed weak cytotoxicity. Meanwhile, no cytotoxicity was detected for Integran in either method. In the MEM elution assay, no cytotoxicity was detected for Seprafilm; however, a moderate degree of cytotoxicity was observed in the colony assay, indicating that Seprafilm considerably affects the proliferation ability of surrounding cells, even though it does not affect the survivability of surrounding cells. The proliferation ability of cells

is more critical for the healing of surgical incisions, so this may reveal the reason for the adverse effects caused by Seprafilm.

Furthermore, the results of the MEM elution assay revealed that the cytotoxicity of Surgicel 2 is higher than that of Surgicel 1. However, the colony assay revealed that the cytotoxicity of Surgicel 1 is higher than that of Surgicel 2. Similar results were also observed for Avitene and Seprafilm. Seprafilm showed no cytotoxicity in the MEM elution assay, and Avitene showed the strongest cytotoxicity among Seprafilm, Avitene, and Integran. However, in the colony assay, much stronger cytotoxicity was observed for Seprafilm than for Avitene. These results suggest that there must be different mechanisms by which biomaterials affect the survivability and proliferation ability of cells. Therefore, we consider that for assessing the cytotoxicity of biomaterials, it is necessary to use a combination of two or more sensitive and quantitative methods to test the effect of biomaterials on both the survivability and proliferation ability of cells. Methods focusing on testing either the effects on the survivability of cells alone or testing the effects on the proliferation ability of cells alone are not adequate for completely revealing the cytotoxicity of biomaterials at the preclinical stage, which is critical for reducing the clinical risks.

In clinical use, absorbable hemostats and adhesion barriers are always utilized as plomb and are in contact with the surgical incision for a long time. The proliferation ability of cells is critical for the healing of surgical incisions, and in this respect, understanding the correlation between the extraction period and the degree of cytotoxicity of an absorbable hemostat or adhesion barrier through colony assays may provide a way to predict prolonged in vivo cytotoxic effects. Thus, the increasing cytotoxicity with increases in the extraction period observed in this study suggests that these surgical materials (Surgicel 1, Avitene, and Seprafilm) can affect the surrounding cells, tissues, or organs during long utilization after a surgical procedure. Based on a comprehensive understanding of the cytotoxic effects of each biomaterial, in clinically utilizing biomaterials, we consider it to be preferable to choose an appropriate material with a suitable utilization method according to the practical surgical conditions.

Conclusion

The cytotoxicities of three kinds of commercially available absorbable hemostats and one adhesion barrier were assessed by two different cytotoxicity testing methods. The different testing methods detected different degrees of cytotoxicities for the materials studied. Cytotoxicity was detected for Seprafilm in the colony assay, whereas no cytotoxicity was detected in the MEM elution test. To the best of our knowledge, this is the first report in which cytotoxicity has been detected for Seprafilm in vitro. We consider that the very low IC_{50} value in the colony assay may provide an answer to the clinical problems that can result from the use of Seprafilm. In conclusion, a combination of two or more sensitive and quantitative methods for safety evaluation is necessary to avoid false-negative results for biomaterials at the preclinical stage. We propose that investigation of the correlation between the cytotoxicity and extraction period may provide a way to predict the prolonged in vivo toxicity of absorbable biomaterials.

Acknowledgments This work was partially supported by Health and Labor Sciences Research Grants for Research on Advanced Medical Technology and Risk Analysis Research on Food and Pharmaceuticals from the Ministry of Health, Labor and Welfare of Japan, as well as Health and Labor Sciences Research Grants for Research on Health Sciences Focusing on Drug Innovation by the Japan Health Sciences Foundation.

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視力補正を目的としないおしゃれ用カラーコンタクトレンズの細胞毒性

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Cytotoxicity of Various Non-corrective and Decorative Contact Lenses

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Abstract

Non-corrective and decorative contact lenses can be purchased as sundries without the guidance of medical doctor. Recently, many cases of eye injury by utilizing these lenses have been reported. To estimate their cytotoxic potential to cause eye injury, cytotoxicity tests of the commercially available non-corrective and decorative contact lenses were performed utilizing the V79 cell colony assay. By the colony assays of the lenses and their extracts, it was suggested that two tested lenses out of the ten are cytotoxic. Although preservatives for these lenses in the products showed cytotoxicity, the cytotoxicity of the two lenses is suggested to be ascribed to un-identified materials, which can be extracted to a cell culture medium from them. The results of this study indicate that cytotoxicity of the non-corrective and decorative contact lenses would be better to be evaluated for estimating their biological safety.

Keywords: non-corrective and decorative contact lens, cytotoxicity, extract, preservative

1. 緒言

現在，視力補正を目的としない場合，すなわちファッション目的で目の色を変えるためのおしゃれ用カラーコンタクトレンズには，薬事法が適用されず規制対象外となっているため，インターネットショップや雑貨店を通じた購入が可能となっている．しかしながら，目に装用して使用するという使用方法は，視力補正を目的としたものと同様である．よって，コンタクトレンズの使用未経験者が専門家の適切な指導を受けないまま，また，それら製品の適切な安全性評価・管理がなされないままそれらのレンズを使用するケースが数多く存在する．PIO-NET（全国消費生活情報ネットワーク・システム）には，おしゃれ用カラーコンタクトレンズを用いた結果生じた炎症や角膜障害などの不具合を中心とした申し出情報が寄せられている[1)]．また，米国においても同様の障害が報告されている[2)]．

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視力補正を目的としないものであっても，コンタクトレンズである以上，本来はその適切な装用法や管理手段の指導を受けるべきであり，報告された不具合はその不適切な使用法が一因であることが大いに考えられる．加えて，その製造や販売にあたり使用部位を考えた場合に行うべき安全性評価・管理がなされないまま，流通していることも予想される．すなわち，PIO-NETに報告されている不具合の原因がおしゃれ用カラーコンタクトレンズ自体の生物学的安全性欠如であることも予想される．

そこで，本研究では，現在入手可能で，ある程度その製造者や材料などが特定できるおしゃれ用カラーコンタクトレンズ10種類を対象として，それらが眼組織に障害を与える可能性を検討するために，眼組織刺激性との相関が報告されている細胞毒性[3)]を評価する試験を行ったので，それらの結果について報告する．

2. 実験

2. 1 試料

今回対象としたおしゃれ用カラーコンタクトレンズをTable 1に示す．対象としたレンズは，全て国内で購入可能なものであり（個人輸入を含む），青系4銘柄，茶系4銘柄，それらの色以外の2銘柄，合計10銘柄を入手した．

Table 1. A list of non-corrective and decorative contact lenses utilized for this study

Sample No.	Color	Made in	Materials and Water contents	Basecurve (mm)	Diameter (mm)	Su
1	Blue	South Korea	Polymacon : 62% Water : 38%	8.6	14	
2		South Korea	PHEMA Water : Unknown	8.6	14	
3		Un-identified*	Co-polymer : 58% (HEMA & MMA) Water : 42%	8.6	14	
4		Taiwan	PHEMA Water : Unknown	8.6	14	
5	Brown	UK	Methafilcon : 45% Water : 55%	8.7	14.4	
6		Un-identified*	Co-polymer : 58% (HEMA & MMA) Water : 42%	8.6	14	
7		UK	Filcon 1B : 45% Water : 55%	8.8	14.4	
8		Singapore	HEMA Co-polymer : 45% Water : 55%	8.6	14.2	
9	Violet	UK	Filcon 1B : 45% Water : 55%	8.8	14.4	
10	Red	UK	Filcon 1B : 45% Water : 55%	8.8	14.4	

* "Materials from USA" is only described on the package of the product.

HEMA: 2-Hydroxyethyl methacrylate
MAA: Methacrylic acid
PHEMA: Poly HEMA
Polymacon: PHEMA (USAN)
Methafilcon A, Etafilcon A: Copolymer of HEMA and MAA (USA
Filcon 1B: ISO category of hydrogel material made from copolyme
Phemfilcon A: Copolymer of HEMA and EOEMA (USAN)
EOEMA: 2-Ethoxyethyl methacrylate

2. 2 細胞毒性試験

細胞毒性試験は，厚生労働省の生物学的安全性試験ガイドライン[4]，ISO関連文書[5]及び細胞毒性試験に関する報告[3, 6, 7]を参考にして，承認申請時に求められるものに準拠した方法で行った．細胞はChinese hamster lung fibroblasts（V79細胞）を用い，Eagle's minimal essential mediumに10 %のウシ胎児血清（FCS）を加えたもので，37° C，5 % CO2培養器中で継代したものを実験に使用した．

具体的な細胞毒性試験手法を以下に示す．試験には，Earleの平衡塩類溶液培地に非必須アミノ酸，ピルビン酸ナトリウム（0.11 g/l），L-グルタミン（0.292 g/l），炭酸水素ナトリウム（2.2 g/l），ペニシリン・ストレプトマイシン及びFCS 5 %を含んだ培地（M05培地）を使用した．24-well プレート（Corning Inc., Corning, NY）に静置した孔径0. 4 μmの tissue culture insert（Nunc A/S, Rocklide, Denmark）中にV79細胞を約25個懸濁させた500 μlのM05培地を加え，さらにinsertを入れたwellに500 μlのM05培地を加え4時間培養して細胞をinsertに接着させた．検体となるコンタクトレンズは，予め別の24-wellプレート中に静置して準備しておいた．V79細胞が接着したinsertを内部の培地を除いた後に速やかに検体入りのプレートに移し，well中及びinsert中にそれぞれ500 μl，合計1 mlのM05培地を加えてさらに培養を行った．1週間後，Giemsa染色を行い，染色されたコロニー数を計測した．なお，試験の検出感度と精度を確認する目的で，細胞毒性試験用の陰性対照材料及び陽性対照材料を用いての試験も同時に行った．陰性対照材料としては組織培養用プラスチックシート（和光純薬製）を，陽性対照材料としては0.1 % zinc diethyldithiocarbamate（ZDEC）含有ポリウレタンフィルム（PU）（陽性対照材料A），0.25 % zinc dibutyldithiocarbamate（ZDBC）含有PU（陽性対照材料B）を（財）食品薬品安全センター秦野研究所より入手して用いた．コンタクトレンズの細胞毒性は検体の入ってないinsert中でのコロニー数を100 %として，各insert中に観察されたコロニー数を比較することで評価した．

次に，上記の方法で細胞毒性があることが疑われたレンズを中心にいくつかのおしゃれ用カラーコンタクトレンズを選び，滅菌プラスチックチューブにそれらのレンズ各10枚を入れレンズ表面積に対して6 cm^2/mlになる量のM05培地を加えた．それらのチューブを24時間細胞培養器中に静置することでレンズ抽出液を調製した．24-well プレートにV79細胞を50個/well/500 μl M05培地で播種して4時間培養を行った後に培地を捨て，調製したレンズ抽出液をM05培地で段階希釈したものを500 μl加えてさらに培養した．1週間培養後に形成されたコロニー数を計測し，抽出液未添加の場合に計測されたコロニー数と比較することで調製したレンズ抽出液の細胞毒性を評価した．

また，各製品のパッケージ中にレンズ保存用としてレンズと共に入っていた保存液の毒性は以下の方法で評価した．24-wellプレートにV79細胞を50個/well/500 μl M05培地で添加して4時間培養後培地を捨て，回収した保存液を添加して段階希釈したM05培地を加えて1週間培養後の形成コロニー数からその毒性を評価した．抽出液及び保存液の試験では，前記2種類の陽性対照材料A及びB，陰性対照材料に加えて陽性対照物質であるZDBCとを試験系の感度及び精度確認のために使用した．

なお，得られたデータはDunnettの多重比較による検定を行い，検体を加えていないコントロール群との有為差を必要に応じて示した．

3. 結果及び考察

コンタクトレンズの使用法を考慮した場合，細胞と直接接触させてその細胞毒性を検討する直接接触法を行うことが理想ではあるが，今回の対象レンズが全てソフトコンタクトレンズのため細胞接着性が低いこと，またレンズ自体は平面でなく眼球に合わせた曲率を持ち材料への均一な細胞播種が不可能であるため，実際のコンタクトレンズ承認申請時にも使用されているtissue culture insertを利用した試験を行った．

Table 2に，コロニー法を用いて算出した細胞毒性試験の結果を示す．