into the growth plate. At present, however, a potential role of HMGB1 as a nuclear factor, which is its other function, still remains possible in the developing cartilage.

Secretion of HMGB1 during specific stages of cell differentiation is not unique to chondrocytes and has been reported for dendritic cells (14) and neonatal rat type I astrocytes (41), although the mechanism of HMGB1 secretion during cell differentiation has yet to be elucidated. Thus far, Bonaldi et al. have reported that HMGB1 contains two nuclear localization signals (NLSs), and the acetylation of both NLSs is involved in the transport from the nucleus to the cytosol (8). Furthermore, HMGB1 can be phosphorylated, and the direction of transport is regulated by phosphorylation of both NLS regions (58). These findings suggest that HMGB1 release is independent from RNA expression and protein synthesis, which is compatible with our data showing that HMGB1 mRNA levels do not change in chondrocyte pellet cultures, despite its secretion.

The inhibition of the interaction between HMGB1 and the receptor for advanced glycation end products (RAGE), which is a specific receptor for HMGB1, suppresses the tumor proliferation, metastatic invasion, and expression of MMPs (48). RAGE is expressed in osteoclasts, osteoblasts (12), and endothelial cells (9), suggesting that RAGE might be associated with cell invasion during endochondral ossification; however, an analysis of *Rage*$^{-/-}$ mice (1) showed no alteration in skeletal development during embryogenesis (see Fig. S1B in the supplemental material). Moreover, *Rage*$^{-/-}$ mice manifest increased bone mass and bone mineral density and decreased bone resorptive activity due to a defect in osteoclast function (60). In our hands, however, MMP9 mRNA levels in calvariae at E18.5 were similar between wild-type and *Hmgb1*$^{-/-}$ mice (see Fig. S3A in the supplemental material), and MMP9-positive cells emerged in the bone marrow of developing limbs of both types of mice at E18.5 (see Fig. S3B in the supplemental material). The evidence that HMGB1-RAGE interaction is sufficient but not necessary for mesoangioblast migration (38) is a precedent for the idea that RAGE may not be the key receptor for HMGB1-induced cell recruitment at the primary ossification center. Additional HMGB1 receptors have been identified, including Toll-like receptors 2 and 4 (39), which appear in osteoclasts, osteoblasts, and endothelial cells (26, 49, 50), and syndecan (43), which is expressed in osteoblasts (24).

Our results indicate that HMGB1 might be important not only for tissue repair after injury but also for the organization of bone and cartilage development in the embryo. In endochondral ossification, a region of resting chondrocytes transforms into a zone of proliferating chondrocytes that then undergo hypertrophy and subsequently apoptosis (37). HMGB1 release from the hypertrophic cartilage occurs just before programmed cell death (Fig. 10), suggesting that HMGB1 may be an extracellular signal released from the tissue to be replaced (cartilage) toward the cells of new tissue to be formed (bone and bone marrow).

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Intraocular Pressure Elevation after Injection of Triamcinolone Acetonide: A Multicenter Retrospective Case-Control Study

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• PURPOSE: To determine the risk factors for intraocular pressure (IOP) elevation after the injection of triamcinolone acetonide (TA).

• DESIGN: Retrospective interventional case-control study.

• METHODS: SETTING: Multicenter. PATIENT POPULATION: Four hundred and twenty-seven patients. OBSERVATION PROCEDURES: Intraocular pressure levels after TA treatment by the sub-Tenon capsule injection (STI; 12 mg, 20 mg, or 40 mg), intravitreal injection (IVI; 4 mg or 8 mg), or the combination of STI (20 mg) and IVI (4 mg), and IOP levels after two TA treatments. MAIN OUTCOME MEASURE: Risk factors for IOP levels of 24 mm Hg or higher.

• RESULTS: Younger age (hazards ratio [HR], 0.96/year; $P < .0001$), IVI (HR, 1.89/year; $P < .0001$), and higher baseline IOP (HR, 1.15/mm Hg; $P = .003$) were identified as risk factors. Dose dependency was shown in STI-treated eyes (HR, 1.07/mg; $P = .0006$), as well as after IVI (HR, 1.64/mg; $P = .013$). The combination of STI and IVI was a significant risk factor (HR, 2.27; $P = .003$) compared with STI alone. In eyes receiving two TA treatments, IVI (HR, 2.60; $P = .010$), higher IOP elevation after the first injection (HR, 1.18/mm Hg; $P = .011$), and increased dosage of STI (HR, 1.07/mm Hg; $P = .033$) were risk factors.

• CONCLUSIONS: Younger age, higher baseline IOP, IVI, and increased TA dosage were associated with TA-induced IOP elevation. IOP elevation after repeated TA injection was frequently associated with eyes treated with IVI, high IOP elevation after the first injection, and high doses of STI. (Am J Ophthalmol 2008; 145:676–681. © 2008 by Elsevier Inc. All rights reserved.)

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TRIAMCINOLONE ACETONIDE (TA) IS COMMONLY USED to treat various vitreoretinal diseases. TA limits the impact of corticosteroids on ocular tissues, thereby minimizing the side effects associated with systemic steroid therapy.[1–5] However, many patients who have received intravitreal injection (IVI) of TA or the sub-Tenon capsule injection of TA (STI) encounter intraocular pressure (IOP) elevation,[6–13] which can develop into glaucoma.[14,15] The prevalence of TA-induced IOP elevation is reportedly between 18% and 50%.[7,13,16–19] This wide range of values might be explained by the following: variation between definitions of IOP elevation; the TA dose and the method of administration; whether patients have previously received TA injections; patient background characteristics, including history of glaucoma or ocular hypertension; and administration of steroids. Several reports have suggested an increased prevalence of TA-induced IOP elevation in younger patients.[1,7,15,20] Therefore, we retrospectively investigated the risk factors for IOP elevation in patients receiving TA at six Japanese clinical centers, based on a standardized definition of TA-induced IOP elevation.

METHODS

• PATIENTS: We reviewed the medical records of patients receiving TA by STI (12 mg, 20 mg, or 40 mg), IVI (4 mg or 8 mg), or simultaneous administration by STI (20 mg) and IVI (4 mg) at the following six clinical centers in Japan: Kumamoto University Hospital (Kumamoto), Nagoya City University Hospital (Nagoya), Kagawa University Hospital (Miki), Kobe University Hospital (Kobe), Kagoshima University Hospital (Kagoshima), and Kansai Medical University Hospital (Moriguchi). Data from patients who received TA between April 1, 2002 and March 31, 2006 were included in the analyses. If both eyes were treated with TA, the eye that was treated first was

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TABLE 1. Patient Data Before Triamcinolone Acetonide Treatment in < 24 mm Hg and ≥ 24 mm Hg Groups

Characteristic (n = 427)	Eyes with < 24 mm Hg (n = 377) n (%)	Eyes with ≥ 24 mm Hg (n = 50) n (%)	*p* value
Male gender	228 (60.5)	30 (60.0)	.948
Mean age (years)	65.8 ± 11.2	57.1 ± 17.5	.006*
Diabetes mellitus	203 (53.8)	17 (34.0)	.008*
Hypertension	137 (36.3)	18 (36.0)	.963
Cataract surgery	141 (37.4)	17 (34.0)	.638
Vitrectomy	88 (23.3)	14 (28.0)	.475
IVI included	69 (18.3)	25 (50.0)	<.0001*
Mean IOP at baseline (mm Hg)	13.8 ± 3.1	15.1 ± 3.1	.010*

IOP = intraocular pressure; IVI = intravitreal injection of triamcinolone acetonide.
*$P < .05$.

investigated. The exclusion criteria were as follows: eyes that had received intraocular surgery within three months before TA treatment; eyes with a history of glaucoma or uveitis; eyes that had shown > 21 mm Hg IOP levels; and patients who had been treated with steroids. Eyes treated with a second TA injection within the follow-up period were included in the analyses. If the TA dose administered in the second injection was different from that in the first, the eyes were included in the analysis of the first injection, but excluded from the analysis of the second.

• MAIN OUTCOME MEASURE AND OBSERVATION PROCEDURE: The main aim of this study was to investigate the risk factors for IOP elevation after TA treatment. The IOP levels after TA treatment were derived from patients' medical records. If any ocular surgeries were performed, IOP data from before the surgeries were evaluated. If an additional dose of TA was administered after the first injection, the IOP data at the first TA injection were evaluated until the second injection. The IOP levels were also evaluated between two weeks and a maximum of 12 months after the second injection. The baseline IOP was defined as the IOP level on the day of TA injection or at the last examination before the TA injection. The IOP data were mainly selected from records obtained by measurement using noncontact pneumotonometry. In line with previous reports,[6,15] we defined an IOP of 24 mm Hg or higher after TA treatment as elevated IOP induced by TA treatment. Furthermore, if IOP levels of 24 mm Hg or higher were shown by the noncontact pneumotonometer, they were re-examined using a Goldmann applanation tonometer on a slit-lamp biomicroscope, and the value shown by the tonometer was used as the IOP. Eyes for which the medical records did not indicate whether re-examination by tonometry had been performed were excluded from the study.

The following variables were assessed as potential risk factors for elevated IOP: gender; age; history of diabetes mellitus, hypertension, cataract surgery, or vitrectomy; dose and route of TA administration (12 mg, 20 mg, or 40 mg by STI; 4 mg or 8 mg by IVI; or a combination of 20 mg by STI and 4 mg by IVI); and baseline IOP. These factors were compared between patients with less than 24 mm Hg and those with 24 mm Hg or higher IOP. Potential risk factors for IOP levels of 24 mm Hg or higher after additional treatment were as described above. The maximal IOP minus baseline IOP (ΔIOP) values after the first treatment and the interval between the first and the second treatment were also assessed.

• STATISTICAL ANALYSIS: Data analysis was performed using the JMP version 6 statistical package program (SAS Institute, Cary, North Carolina, USA). The Mann–Whitney *U* test and the Chi-square test (or the Fisher exact test) were used for the univariate analyses. To confirm the effects of the risk factors and identify the hazard ratios (HRs) for TA-induced IOP elevation, multivariate Cox proportional hazards regression analysis was performed. The multivariate factors were selected from among the variants with a probability (*P*) value of less than .30 shown by univariate analysis. A *P* value less than .05 was considered statistically significant.

RESULTS

IN TOTAL, 427 EYES SATISFIED THE STUDY CRITERIA. ALL OF the eligible patients were Japanese. The diagnoses for the TA-treated eyes were as follows: age-related macular degeneration (67 eyes), other choroidal neovascular diseases (34 eyes), retinal vein occlusion (131 eyes), diabetic retinopathy (180 eyes), and other retinal diseases related to cystoid macular edema (15 eyes). Of these, 319 eyes were treated by one TA injection, and 108 eyes were treated with an additional TA injection. In total, 50 (11.7%) of the 427 eyes had an elevated IOP of 24 mm Hg or higher. IOP elevation of 24 mm Hg or above started 0.5

TABLE 2. Risk Factors for Elevated Intraocular Pressure Elevation of ≥ 24 mm Hg After Triamcinolone Acetonide Treatment—Cox Proportional Hazards Analysis

Variable	Hazards Ratio for ≥ 24 mm Hg	*p* value
Model 1: All eyes treated with triamcinolone acetonide injection (n = 427)		
Age (years)	0.96 (0.95 to 0.98)	<.0001*
Diabetes mellitus	0.76 (0.55 to 1.02)	.068
IVI included	1.89 (1.41 to 2.52)	<.0001*
IOP at baseline (mm Hg)	1.15 (1.05 to 1.27)	.003*
Model 2: Eyes with STI only (n = 333)		
Age (year)	0.96 (0.94 to 0.99)	.003*
Diabetes mellitus	0.91 (0.60 to 1.38)	.647
STI (mg)	1.07 (1.03 to 1.12)	.0006*
IOP at baseline (mm Hg)	1.31 (1.13–1.52)	.0003*
Model 3: Eyes with IVI only (n = 57)		
Age (year)	0.98 (0.94 to 1.03)	.393
Diabetes mellitus	0.91 (0.47 to 1.61)	.760
IVI (mg)	1.64 (1.09 to 3.39)	.013*
IOP at baseline (mm Hg)	1.03 (0.85 to 1.27)	.765
Model 4: Eyes with 20 mg of STI or 20 mg of STI plus 4 mg of IVI (n = 201)		
Age (year)	0.95 (0.92 to 0.98)	.003*
Diabetes mellitus	0.75 (0.41 to 1.29)	.306
Plus 4 mg of IVI	2.27 (1.33 to 4.02)	.003*
IOP at baseline (mm Hg)	1.28 (1.07 to 1.55)	.008*

IOP = intraocular pressure; IVI = intravitreal injection of triamcinolone acetonide; STI = sub-Tenon capsule injection of triamcinolone acetonide.
Hazards ratio is shown with 95% confidence interval.
*$P < .05$.

month after the injection in 12 eyes, after one month in nine eyes, after two months in 19 eyes, after three months in nine eyes, and after six months in one eye. Patient data before TA injection for the group with IOP elevation of less than 24 mm Hg and the group with 24 mm Hg or higher are shown in Table 1. The patients within the 24 mm Hg or higher group were younger, were less likely to have a history of diabetes mellitus, had a greater incidence of IVI administration of TA, and had higher baseline IOP values. The multivariate Cox proportional hazards regression showed that younger age (HR, 0.96 per year; 95% confidence interval [CI], 0.95 to 0.98; $P < .0001$), the inclusion of IVI (HR, 1.89; 95% CI, 1.41 to 2.52; $P < .0001$), and higher baseline IOP (HR, 1.15 per mm Hg; 95% CI, 1.05 to 1.27; $P = .003$) were risk factors for IOP elevation (Table 2; Model 1).

We also examined whether IOP elevation after TA injection was dose-dependent. In eyes treated by STI (n = 333), one of 36 eyes (2.8%), six of 164 eyes (3.7%), and 18 of 133 eyes (13.5%) showed IOP values of 24 mm Hg or higher after doses of 12 mg, 20 mg, and 40 mg by STI, respectively. Cox proportional hazards regression analysis of the 333 eyes identified younger age (HR, 0.96 per year; 95% CI, 0.94 to 0.99; $P = .003$), a higher dose administered by STI (HR, 1.07 per mg; 95% CI, 1.03 to 1.12; $P = .0006$), and higher baseline IOP (HR, 1.31 per mm Hg; 95% CI, 1.13 to 1.52; $P = .0003$) as risk factors (Table 2; Model 2). In eyes treated by IVI (n = 57), one of 18 eyes (5.6%) and 14 of 39 eyes (35.9%) were associated with IOP of 24 mm Hg or higher after doses of 4 mg and 8 mg by IVI, respectively. Cox proportional hazards regression analysis of the 57 eyes showed that a higher dose administered by IVI (HR, 1.64 per mg; 95% CI, 1.09 to 3.39; $P = .013$) was a risk factor. However, neither younger age (HR, 0.98 per year; 95% CI, 0.94 to 1.03; $P = .393$) nor higher baseline IOP (HR, 1.03 per mm Hg; 95% CI, 0.85 to 1.27; $P = .765$) were significant risk factors (Table 2; Model 3). Additionally, 10 of 37 eyes (27.0%) were associated with an IOP of 24 mm Hg or higher after simultaneous administration by STI (20 mg) and IVI (4 mg). In eyes treated with 20 mg by STI, or with both 20 mg by STI and 4 mg by IVI (n = 201), younger age (HR, 0.95 per year; 95% CI, 0.92 to 0.98; $P = .003$), the addition of 4 mg by IVI (HR, 2.27, 95% CI, 1.33 to 4.02; $P = .003$), and baseline IOP (HR, 1.28 per mm Hg; 95% CI, 1.07 to 1.55; $P = .008$) were identified as risk factors (Table 2; Model 4).

Of the 108 eyes treated with a second injection, 16 (14.8%) had IOP elevation to 24 mm Hg or higher. Data

TABLE 3. Data Before the Second Triamcinolone Acetonide Treatment in < 24 mm Hg and ≥ 24 mm Hg Groups

Characteristic (n = 108)	Eyes of < 24 mm Hg (n = 92) n (%)	Eyes of ≥ 24 mm Hg (n = 16) n (%)	*p* value
Male gender	53 (57.6)	10 (62.5)	.714
Mean age (years)	63.1 ± 11.0	55.6 ± 17.8	.279
Diabetes mellitus	50 (54.3)	8 (50.0)	.748
Hypertension	42 (45.7)	3 (18.8)	.044*
Cataract surgery	33 (35.9)	6 (37.5)	.900
Vitrectomy	24 (26.1)	5 (31.3)	.667
IVI included	4 (4.3)	4 (25.0)	.004*
Mean IOP at baseline (mm Hg)	13.8 ± 3.0	13.5 ± 2.4	.900
ΔIOP after the 1st injection (mm Hg)	3.0 ± 3.2	5.8 ± 2.1	<.0001*
Interval between 1st and 2nd injections (months)	5.2 ± 3.2	5.1 ± 3.2	.841

IOP = intraocular pressure; IVI = intravitreal injection of triamcinolone acetonide; ΔIOP = maximal IOP minus baseline IOP.
*$P < .05$.

TABLE 4. Risk Factors for Elevated Intraocular Pressure of ≥ 24 mm Hg After Second Triamcinolone Acetonide Injection—Cox Proportional Hazards Analysis

Variable (n = 108)	Hazards ratio for ≥ 24 mm Hg	*p* value
Model 1: All the eyes treated with repeated TA injections (n = 108)		
Age (years)	0.99 (0.95 to 1.02)	.410
Hypertension	0.71 (0.33 to 1.29)	.276
IVI included	2.60 (1.30 to 4.83)	.010*
ΔIOP after 1st injection (mm Hg)	1.18 (1.04 to 1.30)	.011*
Model 2: Eyes with repeated STIs (n = 100)		
Age (years)	1.03 (0.98 to 1.08)	.247
Hypertension	0.82 (0.37 to 1.58)	.557
STI (mg)	1.07 (1.01 to 1.18)	.033*
ΔIOP after 1st injection (mm Hg)	1.45 (1.17 to 1.85)	.0006*

IVI = intravitreal injection of triamcinolone acetonide; STI = sub-Tenon capsule injection of triamcinolone acetonide; ΔIOP = maximal IOP minus baseline IOP.
Hazards ratio is shown with 95% confidence interval.
*$P < .05$.

before the second TA treatment for the group with elevation of less than 24 mm Hg and the group with elevation of 24 mm Hg or higher are shown in Table 3. The 24 mm Hg or higher group included fewer patients with histories of hypertension, more eyes treated with the inclusion of IVI, and higher ΔIOP after the first injection. Cox proportional hazards regression analysis showed that the inclusion of IVI (HR, 2.60; 95% CI, 1.30 to 4.83; $P = .010$) and higher ΔIOP after the first injection (HR,1.18 per mm Hg; 95% CI, 1.04 to 1.30; $P = .011$) were risk factors for IOP elevation after the additional TA injection (Table 4; Model 1). In eyes treated with two STI injections (n = 100), an increased dose administered by STI (HR, 1.07 per mg; 95% CI, 1.01 to 1.18; $P = .033$) and higher ΔIOP after the first injection (HR, 1.45 per mm Hg; 95% CI, 1.17 to 1.85; $P = .0006$) were shown to be risk factors (Table 4; Model 2).

DISCUSSION

THIS STUDY INVESTIGATED THE RISK FACTORS OF IOP ELEvation following topical TA injection. Cox proportional hazards regression analysis of 427 eyes showed that younger age (HR, 0.96 per year; 95% CI, 0.95 to 0.98), TA treatment including IVI (HR, 1.89; 95% CI, 1.41 to 2.52), and higher baseline IOP (HR, 1.15 per year; 95% CI;1.05 to 1.27) were risk factors for elevated IOP of 24 mm Hg or

higher. These risk factors were also observed in the 201 eyes treated with either 20 mg by STI or a combination of 20 mg by STI and 4 mg by IVI. TA dose dependency for the frequency of IOP elevation was identified by multivariate analyses for 333 eyes treated by STI (1.07 per mg; 95% CI, 1.03 to 1.12) and 57 eyes treated by IVI (1.64 per mg; 95% CI, 1.09 to 3.39). Moreover, multivariate analyses in eyes after two TA treatments showed that TA treatment including IVI, higher ΔIOP after the first TA injection, and a higher dose administered by STI were risk factors.

Several reports have discussed the rates of IOP elevation after TA injection, and have identified potential risk factors. Retrospective studies examining IVI-induced IOP elevation reported that treatment with 20 mg by IVI induced IOP of more than 21 mm Hg in 112 of 272 patients (41.2%),[1] and that 4 mg by IVI induced IOP elevation by 30% or more in 267 of 528 eyes (50.6%),[12] IOP elevation to 24 mm Hg or higher in 36 of 89 patients (40.4%),[6] and IOP elevation to more than 21 mm Hg, or by more than 5 mm Hg, in 26 of 60 patients (43.3%).[20] These results indicate that higher baseline IOP values[6,12] and younger age[1,20] are risk factors for IVI-induced IOP elevation.

By contrast, retrospective studies of STI-induced IOP elevation showed levels equal to or more than 6 mm Hg, or IOP levels of more than 20 mm Hg, in nine of 49 eyes (18.4%),[13] and IOP elevation of equal to or more than 5 mm Hg in 19 of 43 eyes (44.2%).[8] In our previous retrospective study, 40 mg by STI induced high IOP of 24 mm Hg or above in 26 of 115 eyes (22.6%).[15] Younger age[15] and a history of diabetes mellitus[13] are reported risk factors for STI-induced IOP elevation. However, to determine in detail the influence of risk factors, including the dose and route of TA administration, it will be necessary to carry out statistical analysis on a larger number of eyes treated with TA at multiple clinical centers. In this meta-study, to determine the TA-induced IOP elevation more exactly, we excluded eyes with other risk factors for IOP elevation, such as glaucoma, ocular hypertension, uveitis, steroid administration, and recent histories of intraocular surgery. Moreover, TA-induced IOP elevation obtained using noncontact pneumotonometry was confirmed using a Goldmann applanation tonometer. Taken together, our retrospective results reflect the detailed characterization of TA-induced IOP elevation.

No previous large-scale clinical studies have confirmed the risk factors for TA-induced IOP, or examined the effects of the amount of TA administered and the interaction between STI and IVI. The present study not only confirmed that younger age and higher baseline IOP risk factors,[1,6,12,20] but also revealed that IVI induces IOP elevation more frequently than STI, as well as demonstrating the dose dependency for TA-induced IOP elevation. However, no correlations with gender, medical history of hypertension, diabetes mellitus, cataract surgery, or vitrectomy were observed in the analyses for the risk factors. Although some reports have shown that diabetes mellitus is a risk factor for corticosteroid-induced IOP elevation,[13,21] others have shown that it is not significant. A previous randomized diabetes mellitus clinical trial conducted by Palmberg[22] showed that the history of diabetes mellitus was not associated with glaucoma. Our results seem to agree with this. In addition, it could be speculated that the lens and the vitreous affect the diffusion of TA in the ocular tissue; however, no reports (including our present results) suggest that the histories of cataract surgery and vitreous surgery influence TA-induced ocular hypertension.

Interestingly, IVI and ΔIOP are risk factors for IOP elevation in eyes treated with repeated TA injections. IOP elevation is also frequently associated with a higher dose of repeated STI treatment. There are some reports concerning IOP elevation after repeated TA injection.[6,7,12] A study that retrospectively investigated 43 eyes treated repeatedly with 20 to 25 mg by IVI showed that no eyes with 21 mm Hg or less after the first TA injection exhibited more than 21 mm Hg after the second TA injection.[24] By contrast, another study previously reported that 28 of 43 eyes (65.1%) treated with a second TA injection showed an IOP elevation of 30% or more, which was not observed at the first TA injection.[12] In our present study, 15 of 16 eyes with IOP elevation after the second TA injection did not exhibit IOP elevation after the first TA injection. Our present data appear to agree with the latter study, although it showed that the risk factors for IOP elevation after the second TA injection were higher baseline IOP and male gender.[12]

The study presented here has several limitations. First, it shows the risk factors for IOP elevation and not for TA-induced visual field loss attributable to severe TA-induced ocular hypertension. We could not retrospectively quantify visual field loss in eyes with TA-induced IOP elevation because of the association with retinal macular diseases. Second, we did not statistically analyze the duration of IOP elevation in this study. In total, 44 of 50 eyes with IOP elevation in this study showed reversible IOP elevation, whereas six eyes were associated with persistent ocular hypertension in spite of anti-glaucomatous medical treatments. They were treated with trabeculectomy (two eyes) and trabeculotomy (four eyes), which is a surgical procedure effective for corticosteroid-induced glaucoma.[15,23] The six eyes included three treated with 8 mg by IVI, one treated with 4 mg by IVI, one treated with 4 mg by IVI plus 20 mg by STI, and one treated with 40 mg by STI. Persistent IOP elevation might be associated with IVI or high-dose treatment by STI. Third, it remains to be determined whether glaucoma and ocular hypertension are risk factors, as we excluded patients suffering from these disorders from the present study. Such patients might be more

susceptible to TA-induced IOP elevation. Actually, few cases with past histories of glaucoma and ocular hypertension were treated with TA injection. In our clinical centers, TA injection might have been avoided in the patients associated with glaucoma or ocular hypertension. Fourth, we could not evaluate worldwide differences as we only analyzed data from Japanese patients.

In conclusion, our case-control study indicates that younger patients, those with a higher baseline IOP, and those receiving higher doses of TA or intravitreally administered TA are more susceptible to corticosteroid-induced IOP elevation. Greater IOP elevation after the first injection is associated with frequent IOP elevations after the second TA injection.

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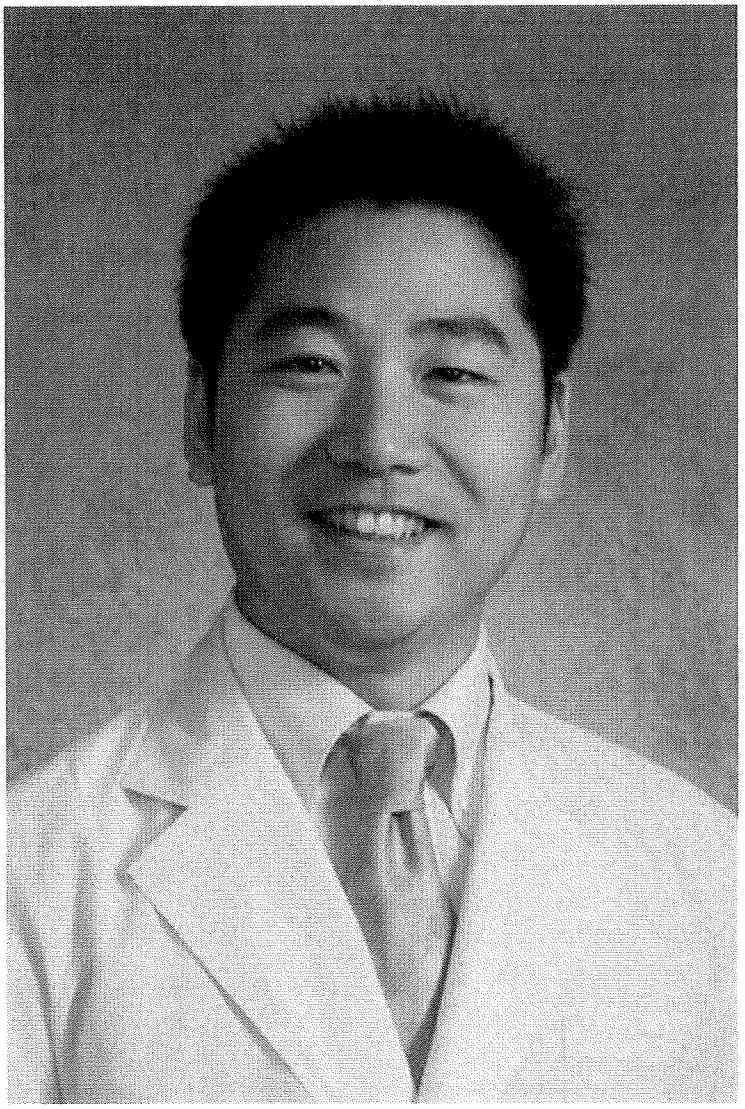

Biosketch

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Research Paper

Inhibition of Melanoma by Ultrasound-Microbubble-Aided Drug Delivery Suggests Membrane Permeabilization

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ABSTRACT

Ultrasound exposure-induced cavitation has been shown to accentuate cell membrane permeability, thus promoting effective drug delivery into cells, a technique that can be enhanced in the presence of microbubbles (MB). Here we applied this method as a treatment for malignant melanoma of the eyelid. The incidence of malignant melanoma in ophthalmology is relatively high, but its treatment is cosmetically difficult. A greater in vitro growth suppression of B-16 melanoma cells was achieved using ultrasound and MB in combination with the anticancer drug bleomycin than when a more concentrated dose of bleomycin alone was applied to the cell culture. Moreover, this effect was enhanced in an in vivo tumor model created by injecting B-16 melanoma cells into the lower eyelids of SCID mice. The antitumor effect of bleomycin was observed at a lower dose (0.5 mg/ml) when the treatment was used in conjunction with ultrasound. The effect was further enhanced when MB were included, with tumor shrinkage occurring at bleomycin levels of 0.06 mg/ml. These results show that ultrasound and MB promote efficient bleomycin uptake by cells, and that the technique is a potentially useful drug delivery method.

INTRODUCTION

In the field of melanoma oncology there continues to be a series of patients that have cancers resistant to standard chemotherapeutic agents. Certainly in other malignancies new chemotherapeutic approaches have demonstrated significant benefit.[1,2] This is less to do with drug efficacy than with the difficulties involved in achieving specific delivery to a target organ while minimizing adverse effects on surrounding healthy tissue.

Ultrasonic therapy has been used for some time in the treatment of cancer.[3-9] In the early stages of its development, attempts were made to destroy cancer cells by using high energy to raise the temperature of the target organ.[10-12] However, this method adversely affected healthy tissue, meaning that its benefits were limited. It has recently been reported that low-energy ultrasound that does not raise tissue temperature, dramatically increases the effectiveness of anticancer agents.[13-16] The mechanism behind this anticancer effect of ultrasound is not well understood, but one possibility is that it causes sonoporation: the development of small pores in the cell membrane caused by the effects of cavitation.[8,17] This low-energy ultrasound method has been used in previous attempts to introduce drugs and genes into cells, but the establishment of the necessary conditions has proved technically difficult.[18,19] If the ultrasound is too weak, transduction efficiency will be low, whereas higher-temperature ultrasound risks damaging healthy cells. For this reason, the method has not reached the stage of clinical application. However, should the resolution of these technical difficulties make its application possible, ultrasound would enable the selective application of drug therapy to target organs with minimal adverse reactions.

Microbubbles (MB), fine bubbles approximately 2-μm in diameter, are used clinically as an ultrasonic contrast medium.[20-22] MB have a unique resonant frequency and, when ultrasound is exposed at that frequency, the MB resonate easily and burst. When ultrasound is irradiated onto a solution containing MB, cavitation will occur, even at frequencies low enough to preclude the risk of damaging cells.[23,24] In this way, it is theoretically possible to deliver substances such as anticancer drugs using ultrasound in a manner that is not harmful to cells.[25-27]

In the present study, we investigated the application of ultrasound of MB as a treatment for malignant melanoma of the eyelid. Melanomas of the eye can involve various ocular structures including the eyelid conjunctiva and uvea, but its treatment is difficult. The reduction of tumor size with this method would therefore be of clinical significance.

MATERIALS AND METHODS

Cell culture. Mouse B-16 melanoma cells (5 x 10^6; ATCC, Manassas, VA) were cultured in modified Eagles's medium (MEM. Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum (FBS. Invitrogen-Gibco, Grand Island, NY) and streptomycin/penicillin (Wako, Osaka, Japan) at 37°C under 5% CO_2. Cells were subcultured at 80% confluency and used between passages P4–P6.

Ultrasound. Ultrasonic conditions were optimized to prevent cell damage. B-16 cells (3.2 x 10^4) were seeded in each well of a 24-well dish with 1 ml MEM supplemented with 10% FBS. A 6-mm diameter Sonitron 2000™ probe (Rich Mar Inc., Inola, OK) was used for ultrasonic irradiation. The probe was inserted directly into the wells of the dish and secured 3 mm above the bottom. Ultrasound irradiation was performed at 1MHz frequency using the following conditions: 1 W/cm^2 power density, 10% duty cycle, 60 s duration; 1 W/cm^2 power density, 50% duty cycle, 60 s duration; 1 W/cm^2 power density, 100% duty cycle, 60 s duration; 1 W/cm^2 power density, 10% duty cycle, 120 s duration; 1 W/cm^2 power density, 50% duty cycle, 120 s duration; 1 W/cm^2 power density, 100% duty cycle, 120 s duration; 2 W/cm^2 power density, 10% duty cycle, 60 s duration; 2 W/cm^2 power density, 50% duty cycle, 60 s duration; 2 W/cm^2 power density, 100% duty cycle, 60 s duration; 2 W/cm^2 power density, 10% duty cycle, 120 s duration; 2 W/cm^2 power density, 50% duty cycle, 120 s duration; 2 W/cm^2 power density, 100% duty cycle, 120 s duration. Cell counts were taken 24, 48, 72 and 96 h after irradiation and the cell growth curve compared with a control group that was not ultrasonically irradiated.

Microbubbles. Cells were diluted to 3.2 x 10^4 cells per 100 µl medium. Optison ™ microbubbles (10, 20 or 40 µl; MB; Amersham Health, Princeton, NJ) were added to each sample and mixed for 5 min. Each sample was transferred to one well of a 48-well dish, which was filled up to 500 µl with MEM medium containing 10% FBS. Cell counts were taken 24, 48, 72 and 96 h after the start of culturing and a cell growth curve was plotted. Using the method described above, an Optison mixture was prepared and irradiated at 1 W/cm^2 power density, 50% duty cycle, and 60 s duration, followed by 2 W/cm^2 power density, 50% duty cycle and 60 s duration. Damage to cells when the MB and ultrasound were used in combination was studied using cell counts and growth curve comparisons as described above. Cells were assessed 24, 48, 72 and 96 h after irradiation.

Effects of bleomycin according to dose method. Bleomycin only. Cells were adjusted to 3.2 x 10^4 cells per well of a 24-well dish. Bleomycin (Nippon Kayaku, Tokyo, Japan) was dissolved in MEM media and adjusted to 5, 0.5, 0.05, 0.005, 0.0005 and 0.00005 µM. Each solution (1 ml) was added to each well. Cell counts were taken 24, 48, 72 and 96 h after the start of culturing and a cell growth curve was plotted. After 24 h, the culture medium was replaced with MEM containing 10% FBS. Experiments were repeated at least three times.

Ultrasound and bleomycin. Six cell/bleomycin mixtures were prepared as above. Ultrasonic irradiation was carried out at 1 W/cm^2 power density, 50% duty cycle and 60 s duration. These conditions did not cause any damage to cells. Cell counts were taken 24, 48, 72 and 96 h after irradiation.

Ultrasound and MB and bleomycin. Six cell/bleomycin mixtures were prepared with 3.2 x 10^4 cells per 100 µl. Optison (5, 10 or 20 µl) was added to one tube of each sample and stirred for 5 min. Each sample was then transferred to one well of a 24-well dish and culture medium added to a total of 1 ml. Ultrasonic irradiation was performed at 1 W/cm^2 power density, 50% duty cycle and 60 s duration. Cell counts were taken as described above.

Calculation of cell count. Cells were counted from digital photographs (IX71, Olympus, Tokyo, Japan) at three random sites per well using NIH Image analysis software (Bethesda, MD) by an examiner who was blind to the experimental details. The mean value for two subjects was regarded as the cell count for each condition. The cell counts were plotted and a cell growth curve drawn. At least three experiments were performed for each condition.

Animals. Five-week-old male SCID mice (n = 5) were anesthetized with an intraperitoneal injection of a mixed solution of ketamine hydrochloride (Ketalal, 60 mg/kg) and medetomidine (Domital, 0.3 mg/kg). After obtaining the approval of the Kagoshima University board, all animal experiments were conducted humanely in strict compliance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research.

P4-5 B-16 cells were harvested and diluted in PBS to a concentration of 1 x 10^6 cells per 50 µl. Under anaesthetic, a 2 x 2-cm area of hair extending from the lower left eyelid to the periphery of the auricle was shaved. Next, 1 x 10^6 cells were injected with a 30G needle using a magnifying glass. Observations were made every two days after the injection, and the experiment commenced when the diameter of the largest tumor reached 4–5 mm.

Bleomycin was injected into the center of tumor using a syringe with a 30 G needle. Immediately thereafter, a 6-mm US probe was placed directly on the tumor surface and US was generated. Every two days after the start of each experiment, each animal's weight and the diameters of the smallest and largest tumors were measured. The estimated tumor weight and mean relative tumor weight were calculated based on the following formulas[28]: Estimated tumor weight = largest diameter (mm) x smallest diameter (mm)^2 x 0.5. Mean relative tumor weight = estimated tumor weight at each measurement point / estimated tumor weight at the start of administration.

The bleomycin anti-tumor effect was evaluated as the index of the mean relative tumor weight.

Evaluation of bleomycin anti-tumor effect. Bleomycin was dissolved in PBS to a concentration of 2 mg/ml and two-fold serial dilution of 1, 0.5, 0.25, 0.125 and 0.06 mg/ml were prepared.

Mice (n = 5–7) were injected with 50 µl bleomycin at 2, 1, 0.5 and 0.25 mg/ml on days 0, 2, 4 and 6. The tumor size and weight were measured two times before injection on days 0–6 and every second day after completion of the injections.

Next, the anti-tumor effects of delivering bleomycin in combination with ultrasound were investigated. Ultrasonic irradiation was performed at 2 W/cm^2 power density, 50% duty cycle, and 240 s duration. These conditions had been shown in previous experiments to cause virtually no tissue damage.[23] Mouse tumors were injected with 50 µl bleomycin at 1, 0.5, 0.25, 0.125 and 0.06 mg/ml prior to ultrasonic irradiation directed onto the tumor site using the 6 mm Sonitron 2000™ probe. The number of injections and tumor size were calculated as described above.

Next, the anti-tumor effects of bleomycin, administered in combination with both ultrasound, and MB were investigated. Ten microliters MB was added to 50 µl bleomycin at 0.5, 0.25, 0.125 and 0.06 mg/ml, and each solution was mixed slowly for 1 min. The mixtures were injected into the tumor, which was then ultrasonically irradiated at 2 W/cm^2 power density, 50% duty cycle, and 240 s duration. Injections and measurements were performed as described earlier.

Table 1 **Optimal conditions of ultrasound irradiation for B-16**

Power	Duty Cycle	Duration	24 h	48 h	72 h	96 h
1 W/cm^2	10%	60 sec	425.00 ± 38.19	516.67 ± 72.65	565.00 ± 21.79	783.33 ± 65.09
		120 sec	360.00 ± 44.44	400.00 ± 57.74	550.00 ± 104.08	866.67 ± 72.65
2 W/cm^2		60 sec	373.33 ± 14.53	466.67 ± 33.33	616.67 ± 60.09	916.67 ± 60.09
		120 sec	466.67 ± 72.65	575.00 ± 43.30	700.00 ± 50.00	816.67 ± 72.65
1 W/cm^2	50%	60 sec	541.25 ± 49.48	831.25 ± 54.36	2005.00 ± 407.57	5225.00 ± 257.91
		120 sec	520.00 ± 34.94	781.25 ± 62.40	1425.00 ± 283.95	4750.00 ± 542.76
2 W/cm^2		60 sec	492.86 ± 49.75	795.71 ± 45.71	1500.00 ± 283.68	4507.14 ± 469.10
		120 sec	493.33 ± 103.49	783.33 ± 94.05	1366.67 ± 72.65	4383.33 ± 60.09
1 W/cm^2	100%	60 sec	75.00 ± 14.43	100.00 ± 0.00	91.67 ± 8.33	116.67 ± 30.05
		120 sec	100.00 ± 14.43	83.33 ± 16.67	83.33 ± 16.67	83.33 ± 16.67
2 W/cm^2		60 sec	83.33 ± 16.67	75.00 ± 14.43	75.00 ± 14.43	83.33 ± 16.67
		120 sec	56.67 ± 9.28	58.33 ± 8.33	58.33 ± 8.33	58.33 ± 8.33
	control		605.75 ± 77.43	863.50 ± 85.34	2460.00 ± 380.08	5906.25 ± 211.38

mean ± S.E.

As a control for the three experiments described above, SCID mice were injected with 50 µl PBS and weight and tumor size measured every two days from the start of the experiment.

Evaluation of ultrasound/optison anti-tumor effects. To evaluate the effects of ultrasound alone on the efficacy of drug delivery, 50 µl PBS was injected into each tumor, which was then ultrasonically irradiated four times at 2 W/cm^2 power density, 50% duty cycle, and 240 s duration. The size of the tumor was measured two times before the injection and every second day after the completion of the experiment. To evaluate the effects of MB alone, a mixture of 10 µl Optison and 50 µl PBS was injected into the tumor, which was irradiated ultrasonically and measured for size as before. Each group in the above experiment contained two mice.

Immunostaining. Bleomycin was chelated with iron that was present in the cell, and reactive oxygen species (ROS) were refined as described.[29,30] When iron binds to the iron binding site within bleomycin it captures oxygen and produces a free radical (ROS) which induces an anti-tumor effect by cleaving DNA.[31,32] As a result of the ROS reaction, the production of the free radical 8OHdG within the cell nucleus was closely monitored. In order to verify that the bleomycin migrated into the cell, immunohistochemical staining was performed on extracted melanoma tumor tissue using the mouse 8OHdG polyclonal antibody (Nikken SEIL, Shizuoka, Japan). The immunostaining protocol was performed according to the method of Hattori et al.[33] 8OHdG is also observed in apoptosis during tumor proliferation. Therefore, tumors of 4 mm diameter were injected with bleomycin alone or in conjunction with ultrasonic irradiation or injected with bleomycin and MB in conjunction with ultrasonic irradiation. The mice were then euthanized, the tumors extracted and embedded in paraffin, then sliced into 5-µm sections, immunostained, and examined by fluorescence microscopy (BX51, Olympus, Tokyo, Japan). The control was defined as a tumor extracted when it had reached 4 mm in diameter before rapid enlargement.

Drug localization. Drug localization was performed using FITC-dextran (FD-4, which has the molecular weight closest to that of bleomycin). FD-4 was diluted in PBS and adjusted to 10 mg/ml. Tumors were injected with 50 µl of FD-4 alone (n = 4), or with 50 µl FD-4 mixed with 10 µl MB in combination with ultrasonic irradiation (n = 4). Ultrasound was irradiated at 2 W/cm^2 power density, 50% duty cycle and 240 s duration. Two samples in each group were extracted 2 h and 20 h after irradiation, sliced into 6-µm sections and observed under fluorescence microscopy.

Side effects. The side effect of the present treatment was evaluated systemically and locally. The systemic effect was evaluated by histological study for various organs such as brain, lung, liver, heart on day 14. The local side effect was also evaluated by histological examination. The local temperature was also monitored by thermographic examinations.[23,34] The irradiated site temperature were calculated during ultrasonic irradiation using thermography TH6200 (NEC San-ei Instruments, Tokyo, Japan), at 2 W/cm^2 power density, 50% duty cycle and 240 second duration. In order to examine the relationship between temperature and tissue damage, the skin on the backs of SCID mice was also irradiated at 2 W/cm^2 power density, 50% duty cycle and 240 second duration, and the strongest irradiated energy of 2 W/cm^2, 100% duty cycle and 240 second duration. Twenty-four hours after the completion of the experiment, approximately 2 x 2 cm of skin from the center of the irradiated site was removed with scissors from euthanized animals and histologically examined.

Histopathological examination. After the experiment, each mouse was euthanized with an intraperitoneal overdose injection of pentobarbital sodium salt (Nembutal, 100 mg/kg), and the tumor was extracted and examined histopathologically. Samples for fluorescence microscopy and 8 OHdG immunohistological staining were fixed in formaldehyde (diluted to 3.7% with PBS), fully dewatered with alcohol, paraffin-embedded, and 5-µm sections prepared. Morphological tissue changes were examined by hematoxylin and eosin (HE) staining.

Following tumor resection, the fluorescence microscopy sample of FD-4-treated tissue was immediately frozen in liquid nitrogen and 6-µm sections prepared. FITC fluorescence was observed using a 515-nm filter.

Statistical analysis. All values were expressed as means ± standard error of the mean (SEM). Repeated measure analysis of variance (ANOVA) with a subsequent paired t test was used to determine the significance of differences in a multiple comparison. A p value of <0.05 was considered statistically significant.

RESULTS

Optimal conditions. The ultrasound conditions that would be most effective in drug delivery while avoiding extensive damage to cells were investigated. As per our previous experiment,[23] the cell count 96 h after irradiation was low (approximately 500) and cell growth was similar or significantly lower than the control under several conditions (Table 1). When the duty cycle was set at 50%, there was no significant difference in cell counts between the irradiation times of 60 or 120 s. However, cell damage was more extensive at 120 s.

Following administration of 10, 20 and 40 µl Optison, the cell count was slightly lower than the control although the differences were not significant (data not shown). When ultrasound was irradiated in conjunction with Optison, the cell count was lower at 40 µl than at 10 or 20 µl (data not shown). Experiments thereafter were performed at 1 W/cm^2, 50% duty cycle and 60 s duration, under which conditions ultrasound and MB had little effect on cell counts or growth.

Effects of bleomycin according to dosage. In the group that received a single dose of bleomycin alone at a concentration of 5 µM, the cell count at all measurement points was suppressed at around 500, which was significantly different to the control (Fig. 1A). When bleomycin was administered at concentrations of 0.5, 0.05, 0.005, 0.0005 or 0.00005 µM, the cell count and growth rate did not differ significantly from the control (Fig. 1A).

In the groups receiving bleomycin in combination with ultrasound at 5 or 0.5 µM, the cell count at each observation was suppressed at around 500, which was significantly different to the control (Fig. 1B). When bleomycin was administered at concentrations of 0.05, 0.005, 0.0005 and 0.00005 µM, there were no significant differences from the control in terms of cell count or growth rate (Fig. 1B).

In the group given a combination of MB, bleomycin and ultrasound, cell count suppression was seen at concentrations of 5, 0.5, 0.05, 0.005, 0.0005 and 0.00005 µM when 10 µl MB was administered. The cell count increased with decreasing concentrations of bleomycin (Fig. 1C). Similar results were achieved when 20 µl MB was administered (data not shown).

Evaluation of bleomycin anti-tumor effects. The anti-tumor effects of bleomycin were assessed by comparing mean relative tumor weights. The tumor was described as "enlarged" if its mean relative weight was 1 or higher, and "decreased" if it was less than 1. In the control group, the mean relative tumor weight rapidly increased throughout the experiment (Fig. 2A).

In the group administered with bleomycin only, the mean relative tumor weight increased even when the highest concentration of 2 mg/ml was administered. There was no tumor suppression effect at any of the concentrations studied. Although it was possible to slow the speed of tumor enlargement at 1 mg/ml and 2 mg/ml, the tumors resumed their rapid enlargement when bleomycin administration was stopped (data not shown). This tumor growth suppression effect decreased as the bleomycin concentration was lowered. The speed of enlargement was greatest at bleomycin concentrations of 0.5 mg/ml and 0.25 mg/ml (Fig. 2A, a).

In the group administered with bleomycin in combination with ultrasound, the mean relative tumor weight at 1 mg/ml decreased throughout the experiment (Fig. 2A, b). At 1 mg/ml and 0.5 mg/ml, the mean relative tumor weight at the completion of the experiment and afterwards decreased to 1 or less and the size of the tumor

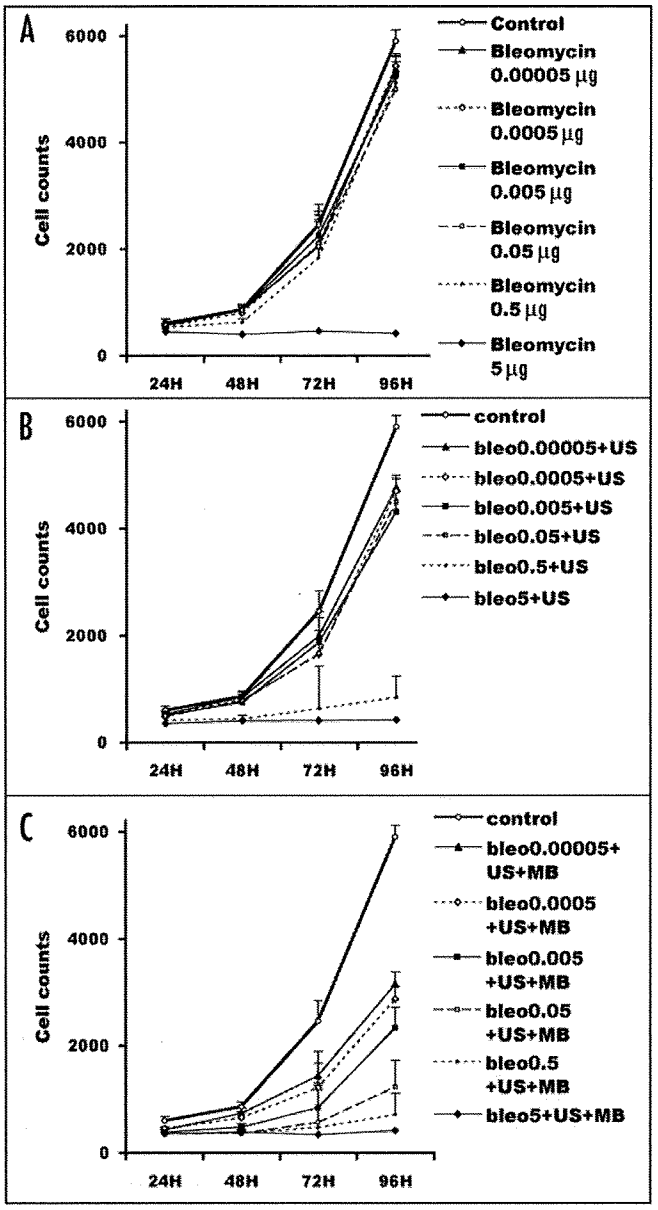

Figure 1. Effects of bleomycin according to in vitro dosage. Cell counts 24, 48, 72 and 96 h after irradiation at concentrations of 5 µM, 0.5, 0.05, 0.005, 0.0005 and 0.00005 µM were compared with controls: for (A) the group receiving a single dose of bleomycin alone (B) the group receiving ultrasound and bleomycin and (C) the group receiving a combination of Optison, bleomycin and ultrasound.

decreased (data not shown). At 0.25 mg/ml, the size of the tumor was virtually unchanged and there was no enlargement after completion of the experiment. At concentrations of 0.125 mg/ml and 0.06 mg/ml, tumors were seen to enlarge to a moderate degree. Although tumor shrinkage was not observed as a result of bleomycin treatment alone at any concentration, significant shrinkage was noted when ultrasound was combined with bleomycin treatment, even at low concentrations of bleomycin.

In the group administered with a combination of bleomycin, ultrasound and MB, there was clear tumor shrinkage at concentrations

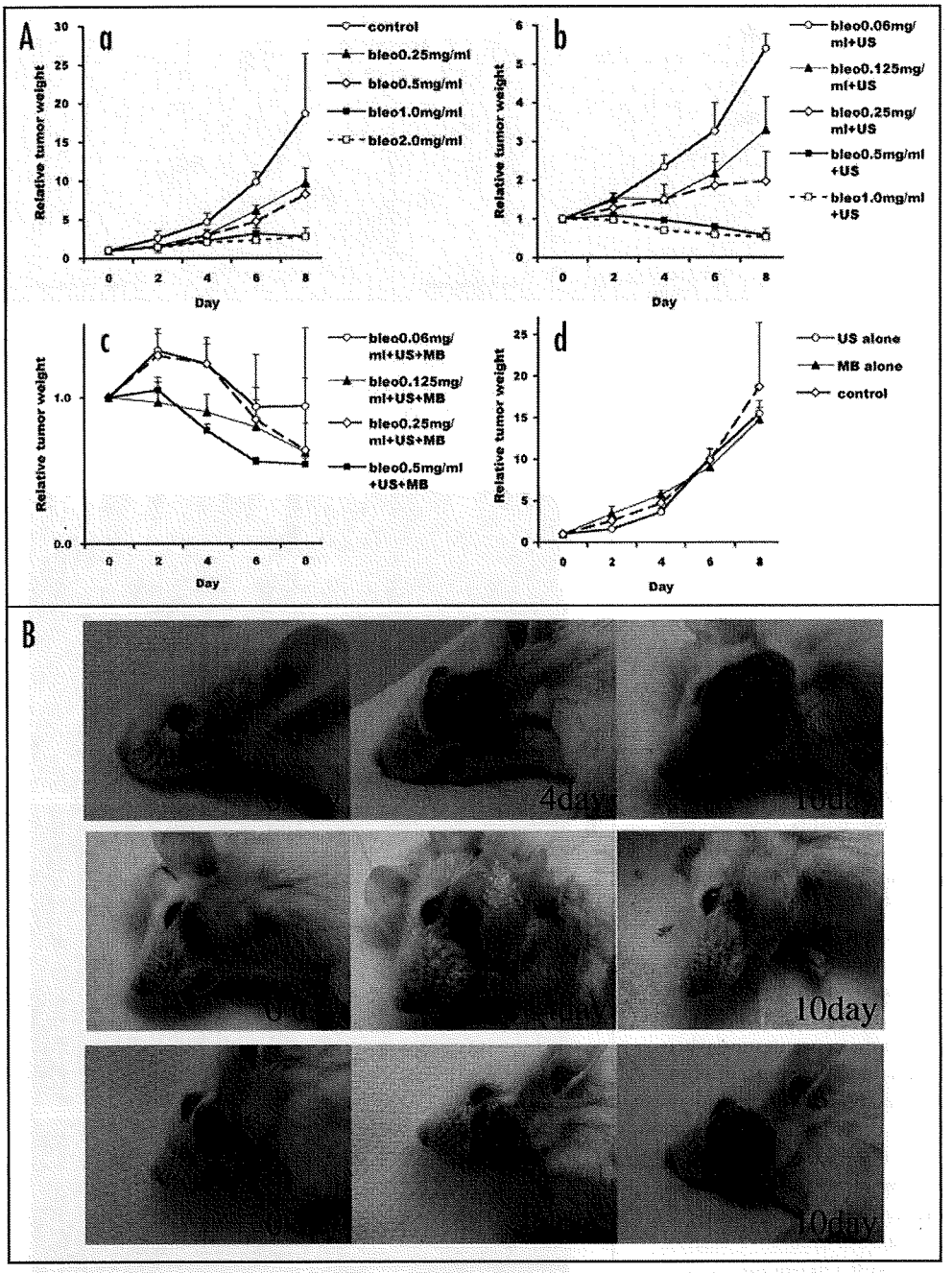

Figure 2. Anti-tumor effects of bleomycin in the malignant melanoma mouse model. (A). The anti-tumor effects of bleomycin were assessed by comparing the mean relative tumor weight of controls with (a) the group administered bleomycin only, (b) the group administered bleomycin in combination with ultrasound, (c) the group that received bleomycin in combination with ultrasound and MB, and (d) the groups receiving either ultrasonic irradiation or MB treatment alone. (B) Examples of the visual observation of tumor growth in the mouse model. A representative animal from each of the groups treated with (a) bleomycin only, (b) bleomycin in combination with ultrasound and MB and (c) ultrasound only is shown. The left panel shows the mice at day 0, when the tumor diameter had reached 4–5 mm, the middle panel after four days, right panel after ten days.

of 0.5 mg/ml (Fig. 2A, c). At 0.25 mg/ml, tumors gradually enlarged but did not increase further after the completion of the experiment. At 0.125 mg/ml and 0.06 mg/ml, tumor enlargement took place, but at a slow rate compared to the control. Thus, when MB were included in the treatment, the extent of tumor shrinkage was greater than that achieved using ultrasound alone at the same concentrations of bleomycin (Fig. 2B).

Evaluation of ultrasound/MB anti-tumor effects. In the groups receiving ultrasonic irradiation or MB treatment alone, no tumor shrinkage was observed and there was no significant difference in terms of cell counts or growth rates from the control (Fig. 2A, d).

Immunostaining. A large number of cells stained positive for 8 OHdG in the group administered with bleomycin in combination with ultrasound, and MB (Fig. 3B) and in the group administered with bleomycin in combination with ultrasound (data not shown). Comparatively few cells in the control group stained positive for 8OHdG (Fig. 3A). The results of immunostaining were consistent with findings within the tumor cell nuclei.

FITC-dextran. Strong, uniform fluorescence of the tumor cell nucleus and cytoplasm and peripheral tissue was observed after 2 h in the FD-4 injection only group, the ultrasound only group, and the ultrasound/MB combined group (Fig. 4A). After 20 h, virtually no fluorescence was seen in the injection only group (Fig. 4B). Strong, patchy fluorescence remained in the ultrasound group and ultrasound/MB group, which was particularly strong on the side of the tumor that had received ultrasound irradiation (Fig. 4C).

Side effects. After the irradiation of ultrasound of 2 W/cm^2 power density, 50% duty cycle, and 240 second duration, the local temperature increased from 34–37°C immediately after irradiation, and remained at 37°C constantly thereafter. The temperature of the probe showed similar changes. Ultrasound irradiation of the backs of SCID mice at 2 W/cm^2 power density, 50% duty cycle, and 240 second duration showed similar results to tumor irradiation, and there were no particular abnormalities in the external appearance during extirpation. At the time of excision, the skin appeared slightly reddish in several places. Hematoxylin and eosin staining of the

excised tissue revealed no abnormalities in the tissue exposed to ultrasound of the duty cycle set at 50%. While, when it came to 100%, some of the epithelial cells were found to have disappeared or the epidemis looked thinned. The histological examination of brain, lung, liver or heart on day 14. The results showed no abnormalities such as inflammation or degeneration in any tissues (data not shown).

Histological examination. In the control group, HE staining revealed active cell division, nuclear heteromorphism and pigmentation, and active proliferation of melanoma cells (Fig. 5A). In the tissue of tumors that had been reduced in size, only a small amount of tumor cells remained in the deepest part of the tumor. This was the case for tumors in the two groups treated with bleomycin in combination with ultrasound, and with ultrasound and MB. Most of these remaining tumor cells had necrotized (Fig. 5B). In cases where tumor enlargement was seen, those groups in which the speed of tumor growth was slower than the control also showed necrosis. The ultrasound only group and MB only group did not differ greatly from the control group, and no abnormalities were seen in the tissue at nontumor sites.

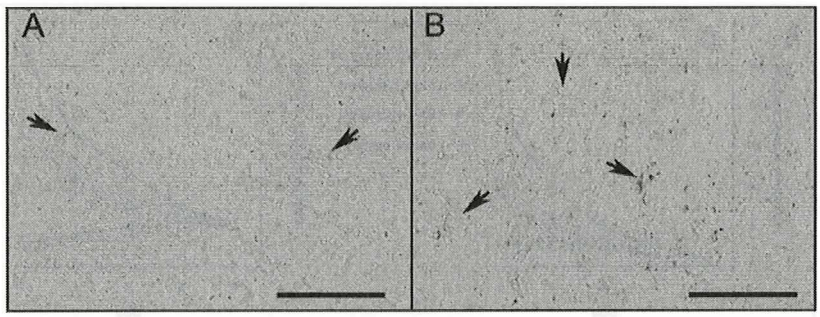

Figure 3. Immunohistochemical staining of the melanoma tumor tissue using the mouse 8 OHdG polyclonal antibody. Arrows indicate cells staining positive for 8 OHdG in (A) the control group and (B) the group administered with bleomycin, ultrasound (irradiation conditions: 2 W/cm^2 power density, 50% duty cycle and 240 sec duration) and MB. Bar, 50 μm.

DISCUSSION

The present study shows that the inhibitory effect of bleomycin on the growth of B-16 cells was significantly enhanced in vitro after ultrasound irradiation alone and when ultrasound irradiation was combined with MB administration. In vivo results showed that following the administration of 2.0 mg/ml bleomycin alone tumors continued to become enlarged gradually, but when bleomycin treatment, even at a concentration of 0.25 mg/ml, was combined with ultrasound, the growth in size of most tumors was arrested. Furthermore, when bleomycin, ultrasound irradiation, and MB were administered concomitantly, tumors decreased in size, even at the low bleomycin dose of 0.06 mg/ml. The same effect as bleomycin treatment alone was therefore achieved at 1/8 the concentration when ultrasound was included, and at a remarkable 1/33 concentration when both ultrasound and MB were used in combination.

Low-energy ultrasound has recently been highlighted as a drug/gene transfer method, but it is unclear why in the present experiment a tumor suppressive effect was enhanced by ultrasound irradiation, and why this effect was further enhanced by adding MB.[5,7,20] In our experiment, tumor suppression was not seen in a control group whose tumors were only irradiated with ultrasound, or in a control group administered with MB alone. The temperature of the irradiated site was 37°C (data not shown), so it is unlikely that the tumor suppression effect was due to the heat of the ultrasound or to a harmful effect of the MB.

Previous reports have demonstrated that small, reversible openings form in the cell membrane due to a cavitation effect caused by low-energy ultrasound irradiation.[35,36] Furthermore, it is known that because MB are similar to the vesicles present during cavitation, a similar effect to ultrasound irradiation occurs, even at lower energy levels.[21,24] MB liquid microjets initiated by rupture from the ultrasound cause small openings to form in the cell membrane. We therefore examined whether ultrasound (alone or in combination with microbubbles) accentuated the intracellular uptake and

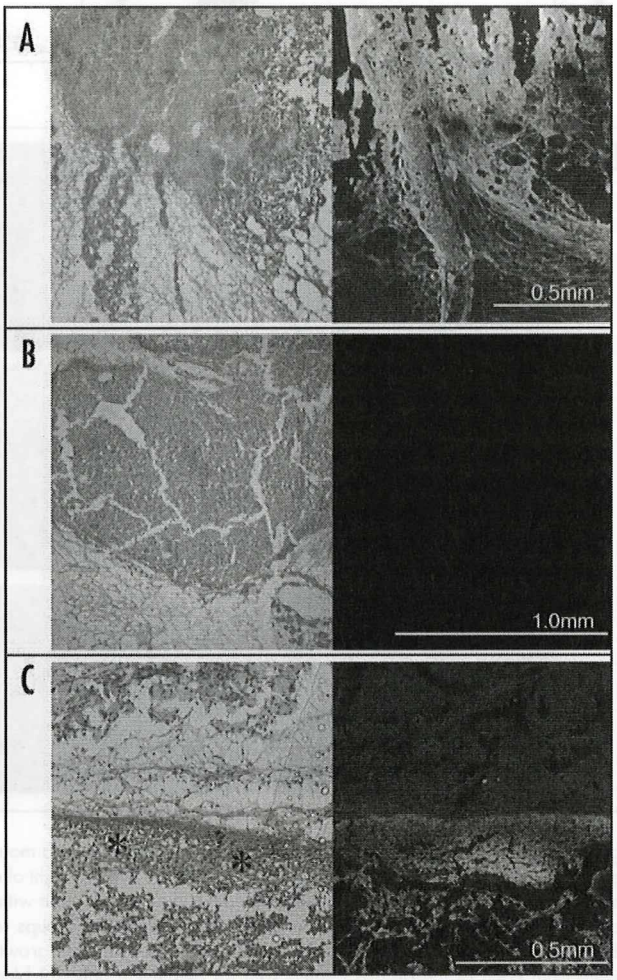

Figure 4. FITC-dextran. Fluorescence microscopy images after FD-4 in (A) the FD-4 only injection group after 2 h, (B) the FD-4 only injection group after 20 h and (C) the ultrasound ultrasound/MB group after 20 h. The right panel shows that the fluorescence in this group was particularly strong on the side of the tumor that had received ultrasound irradiation. Asterisks indicate the side of the tumor.

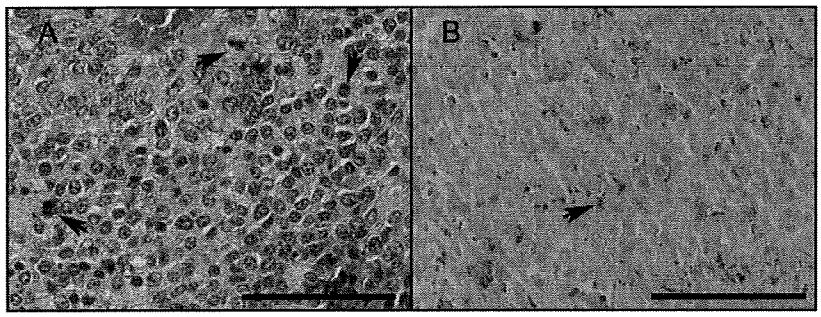

Figure 5. HE staining of extracted tumor tissue. HE staining of tumor tissue samples from (A) the control group and (B) the group treated with bleomycin, ultrasound and MB. Tumor cells are indicated by arrows. Active cell division, nuclear heteromorphism, and pigmentation are evident. Bar, 50 μm.

pharmacological effects of bleomycin. FITC-dextran (FD-4, MW 4000) was used, as we could not directly observe bleomycin.

Our results showed that 20 h after injection of FD-4 alone, virtually no fluorescence remained. When ultrasound and Optison were combined with FD-4 injection, extensive intracellular fluorescence remained 20 h after treatment. That FITC fluorescence was observed throughout the entire cell suggests that dextran entered the cell nonselectively through the cell membrane rather than through selective phagocytosis.

Next, we examined changes that occurred after bleomycin entered the cell. Bleomycin damages DNA by entering the cell nucleus and chelating with iron, thereby producing ROS.[29,31,32] Using this knowledge, immunostaining for 8O HdG was performed to measure damage.[33] The results revealed a high level of positive staining in the cell nuclei in the groups treated with bleomycin and ultrasound only and with ultrasound and MB combined. In contrast, 8OHdG was virtually unseen in the control group and the group administered with bleomycin alone. Although these results provide only indirect proof, they suggest that ultrasound and MB introduce bleomycin into the cell at a high rate, thus producing a pharmacological effect. These findings are consistent with experimental results previously reported.[13,14,17]

Also of note is that the localization of FD-4 and 8OHdG was observed more specifically in melanoma cells than in peripheral healthy tissue. The reason for this is unclear, although it might be that the cell membrane of tumor cells is more sensitive to ultrasound than that of noncancerous cells. Kondo et al. reported that levovist, a type of microbubble, inherently and selectively adsorbs to leukocytes.[37] If microbubbles adsorb to malignant melanoma cells in the same way, this could provide an explanation for the localization of FD-4 and 8 OHdG. This idea is currently under investigation.

In the present study, the ultrasound was not strongly cytopathic in B-16 cells at 1 W/cm^2 power density, 50% duty cycle, and a duration of 120 s or less or at 2 W/cm^2 power density, 50% duty cycle and a duration of 120 s or less. There were also no histological abnormalities in tumor peripheral tissue, and the irradiated site did not reach a high temperature (data not shown).

It is not clear whether the same therapeutic effects would be obtained in humans as observed in the mouse experiment, but we do not anticipate any major problems in clinical applications using the tools and conditions tested in the current study. In the treatment of large facial tumors, cosmetic problems can arise after surgery, even when a tumor is resected. It would therefore be extremely beneficial to resect the tumor after reducing its size using the present method.

In conclusion, the anti-tumor effect of bleomycin on B-16 melanoma cells was enhanced by low-energy ultrasound that was not harmful to tissue. By combining this treatment with microbubbles, the effect was further enhanced and tumor growth was inhibited even at a low concentration of the drug. A nonthermal effect of low energy ultrasound could be an effective method for treating cancer. In particular, it could enable dosages of anticancer drugs to be lowered, and ameliorate adverse reactions. Moreover, the indication that microbubbles are a potentially useful tool for drug delivery should lead to the development of new transfer methods.

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Heparan Sulfate Regulates Self-renewal and Pluripotency of Embryonic Stem Cells*[S]

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Embryonic stem (ES) cell self-renewal and pluripotency are maintained by several signaling cascades and by expression of intrinsic factors, such as Oct3/4 and Nanog. The signaling cascades are activated by extrinsic factors, such as leukemia inhibitory factor, bone morphogenic protein, and Wnt. However, the mechanism that regulates extrinsic signaling in ES cells is unknown. Heparan sulfate (HS) chains are ubiquitously present as the cell surface proteoglycans and are known to play crucial roles in regulating several signaling pathways. Here we investigated whether HS chains on ES cells are involved in regulating signaling pathways that are important for the maintenance of ES cells. RNA interference-mediated knockdown of HS chain elongation inhibited mouse ES cell self-renewal and induced spontaneous differentiation of the cells into extraembryonic endoderm. Furthermore, autocrine/paracrine Wnt/β-catenin signaling through HS chains was found to be required for the regulation of Nanog expression. We propose that HS chains are important for the extrinsic signaling required for mouse ES cell self-renewal and pluripotency.

Embryonic stem (ES)[2] cells from the inner cell mass of preimplantation mouse embryos can be used to establish pluripotent cell lines (1, 2). ES cells retain the ability to differentiate into the representative cell types of all three germ layers of the developing mouse embryo. Human ES cell lines have been derived (3), and the pluripotency of these cells is a feature providing considerable potential for exploitation in the development of cell replacement therapies to treat human disease. However, the molecular mechanisms that control pluripotency and differentiation of ES cells are largely unknown. It will be essential to gain a better understanding of these mechanisms to exploit the potential of ES cells for therapeutic purposes.

A number of studies have investigated the factors controlling pluripotency of mouse ES (mES) cells (4). Self-renewal of mES cells is sustained by signals mediated by the cytokine, leukemia inhibitory factor (LIF) (5, 6). LIF signals through the heteromeric receptor gp130 and the low affinity LIF receptor to induce activation of STAT3 (7–10). However, exposure of cells to serum is also required for LIF-mediated maintenance of self-renewal (11).

Treatment of mES cells with the bone morphogenic proteins (BMPs) BMP2 and BMP4 or with growth differentiation factor 6 can substitute for serum. The ability of BMP/growth differentiation factor 6 to promote self-renewal requires co-stimulation with LIF (11). BMP induces the expression of inhibitor of differentiation (*Id*) genes through activation of Smad signaling, and the *Id* gene products suppress expression of genes involved in the induction of neural differentiation (11). Thus, BMP suppresses neural differentiation and, in combination with LIF, is sufficient to sustain self-renewal of mES cells without the need for feeder cells or serum factors.

Wnt signaling was shown to play a role in the regulation of self-renewal of both mouse and human ES cells independently

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[2] The abbreviations used are: ES, embryonic stem; HS, heparan sulfate; mES, mouse ES; LIF, leukemia inhibitory factor; BMP, bone morphogenic protein; Id, inhibitor of differentiation; FGF, fibroblast growth factor; siRNA, small interfering RNA; MEF, mouse embryonic fibroblast; EB, embryoid body; BIO, 6-bromoindirubin-3′-oxine; MeBIO, 1-methyl-6-bromoindirubin-3′-oxine; CS, chondroitin sulfate; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; AP, alkaline phosphatase; Tcf, T-cell-specific factor; SPR, surface plasmon resonance; vWF, von Willebrand factor; RNAi, RNA interference; PI3K, phosphoinositide 3-kinase; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FACS, fluorescence-activated cell sorter; IL, interleukin; RT, reverse transcription; EGFP, enhanced green fluorescent protein; ERK, extracellular signal-regulated kinase.

of LIF/STAT3 signaling (12). Wnt proteins play roles in the regulation of gene expression, cell proliferation, and differentiation and in the maintenance of cell polarity (13). The binding of Wnt protein to its cognate receptor, Frizzled, results in the inhibition of glycogen synthase kinase-3. This in turn leads to the stabilization and nuclear accumulation of β-catenin and to changes in gene transcription. Signaling by this canonical Wnt pathway has been suggested to result in downstream activation of expression of the homeoprotein Nanog, a transcription factor that is essential for maintenance of the inner cell mass and of ES cell pluripotency (14, 15). The activation of Nanog sustains ES cell self-renewal without the use of feeder cells or treatment with LIF (12).

Heparan sulfate (HS) proteoglycans are ubiquitously present in the extracellular matrix and on the cell surface. The HS polysaccharide chains of the proteoglycans are covalently attached to several core proteins (16). HS chains are synthesized in the Golgi apparatus by several enzymes, including members of the EXT protein family. The chains consist of repeating disaccharide units of D-glucuronic acid-*N*-acetyl-D-glucosamine that are modified differentially by epimerization and sulfation (16). A large number of physiologically important molecules can bind to specific sulfated regions of HS chains (17). Genetic studies have shown that HS chains regulate biological functions by interacting with various extracellular signaling molecules, such as members of the fibroblast growth factor (FGF) family, Wnt/Wingless (Wg), Hedgehog (Hh), and BMP (18). In *Drosophila*, for example, analyses of mutations of the *EXT* family genes *tout-velu* (*ttv*), *sister of ttv* (*sotv*), and *brother of ttv* (*botv*) have indicated that HS chains are required for signaling and distribution of Hh, Wg, and Decapentaplegic (the functional ortholog of mammalian BMP2 and BMP4) during embryogenesis and wing development (19–22). In mammals, the importance of HS chains in development has been demonstrated by analyses of mutations of enzymes required for HS chain modification, and FGF and Indian hedgehog signaling through HS chains has been suggested to be required during development (18, 23–26). Thus, there is evidence that HS chains have essential functions in development, however, it is not yet clear what role HS chains play in the regulation of early embryogenesis and in ES cells.

Our current understanding suggests that HS chains may contribute to the maintenance of ES cell self-renewal by regulating the activities of several signaling pathways, such as LIF/STAT3, BMP/Smad, and Wnt/β-catenin. In the present study, we investigated the contribution of HS chains to the regulation of ES cell self-renewal and pluripotency. We used small interfering RNA (siRNA) to knockdown *EXT1*, which is required for HS chain elongation. Transfected mES cells grew more slowly than untreated control cells and differentiated into extraembryonic endoderm even in the presence of LIF and serum. This is the first demonstration of the importance of HS chains for the maintenance of self-renewal and pluripotency of mES cells.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—R1 (27) and E14TG2a (28) mES cell lines were maintained on mouse embryonic fibroblasts (MEFs) inactivated with 10 μg/ml mitomycin C (Sigma) in ES medium (Dulbecco's modified Eagle's medium supplemented with 15% FBS {Hyclone}, 1% penicillin/streptomycin {Invitrogen}, 0.1 mM mercaptoethanol {Invitrogen}, and 0.1 mM nonessential amino acids {Invitrogen}) with 1000 units/ml LIF (Chemicon). We generated siRNA expression plasmids targeting *EXT1* and *EGFP* as negative control by inserting of the corresponding double strand DNAs into the BamHI-HindIII site of pSilencer 3.1-H1 (Ambion). The sequences used for RNAi were designed as described previously (29): *EGFP*, 5′-GATCCCGCCACAACGTCTATATCATGGGGAAAATCCATGATATAGACGTTGTGGCTTTTTTGGAAA-3′; *EXT1-1*, 5′-GATCCCGTCCTACCGCAGTATTCATCTGCTTCCTGTCACAGATGAATACTGCGGTAGGACTTTTTTGGA-3′; *EXT1-2*, 5′-GATCCCGGTCTATTCATCAGGATAAAAGCTTCCTGTCACTTTTATCCTGATGAATAGACCTTTTTTA-3′. For transient knockdown of *EXT1* mRNA by RNAi, siRNA expression plasmids for *EXT1* were transfected into mES cells as follows. Prior to transfection, the mES cells were harvested, replated at 1×10^6 cells on gelatin-coated feeder-free 60 mm tissue culture dishes (Iwaki) in ES medium with LIF, and incubated for 16 h. On day 1, the cells were transfected with an siRNA expression plasmid (2 μg per culture dish) using Lipofectamine 2000 (Invitrogen). On day 2, the cells were harvested and replated at 3×10^6 cells on gelatin-coated feeder-free 60 mm tissue culture dishes in ES medium with LIF and 2 μg/ml puromycin (Sigma). In general, puromycin selection of transfected cells was carried out for 24 h. On day 3 (2 days after transfection), the cells were harvested and analyzed as described below.

For differentiation into embryoid bodies (EBs), the cells were transferred on day 3 to low-cell-binding 60 mm dishes (Nunc) and cultured in ES medium without LIF. The numbers of small noncystic EBs and large EBs filled with cystic cavity were counted by microscopic examination. EBs were fixed overnight in 4% paraformaldehyde at 4 °C, dehydrated, embedded in paraffin and sectioned at 10 μm. Sections were stained with hematoxylin and eosin (Merck).

For morphological observation and real time PCR analysis of differentiation markers, the cells were replated on day 2 and incubated with puromycin for 3 days.

For exogenous activation of Wnt/β-catenin signaling, the cells were treated with 2 μM 6-bromoindirubin-3′-oxine (BIO; R&D Systems), a specific pharmacological inhibitor of glycogen synthase kinase-3 or 1-methyl-6-bromoindirubin-3′-oxine (MeBIO; R & D Systems), an inactive analog of BIO during transfection and culture.

FACS Analysis—Two days after transfection, mES cells were harvested and the cell suspension was incubated with mouse IgM negative isotype control (Chemicon), anti-HS antibody 10E4 (Seikagaku Corp.) or anti-chondroitin sulfate (CS) antibody 2H6 (Seikagaku Corp.) diluted in FACS buffer (0.5% bovine serum albumin (BSA) and 0.1% sodium azide in PBS). After washing, the cell suspension was incubated with fluorescein isothiocyanate-conjugated secondary antibody (Sigma) diluted in FACS buffer. Cell sorting and analysis were performed using a FACSAria Cell Sorter (BD Biosciences).

High Performance Liquid Chromatography (HPLC) Analysis of Unsaturated Disaccharides—Two days after transfection, mES cells were harvested and fluorometric post-column HPLC analysis of unsaturated disaccharides from HS chains was performed as reported previously (30).

Molecular Size Analysis of HS Chains—One day after transfection, mES cells were harvested, replated at 1.5×10^6 cells per well in 6-well 0.2% gelatin-coated plates and incubated in sulfate-free ES medium with LIF, puromycin and 100 μCi/ml $Na_2{}^{35}SO_4$ (ARC). After labeling for 24 h, the cells were washed twice with PBS and then treated with 1 mg/ml trypsin (WAKO) for 10 min at 37 °C. The trypsin was neutralized with 2 mg/ml trypsin inhibitor (Roche Applied Science). After centrifugation, the supernatants were treated with 0.5 M NaOH at 4 °C overnight and neutralized with 1 M acetic acid. The labeled galactosaminoglycans were eliminated by chondroitinase ABC (Seikagaku Corp.) digestion, desalted in a PD-10 column (GE Healthcare) and resistant HS chains were isolated by anion exchange chromatography on HiTrap DEAE FF (GE Healthcare) using sodium phosphate buffer (pH 6.0) containing 1.0 M NaCl as the eluent. The sizes of the purified HS chains were analyzed by gel chromatography on a Sephacryl S-300 column (GE Healthcare) (1 × 44 cm) and eluted with 50 mM Tris-HCl, pH 7.4, containing 0.2 M NaCl. Fractions (1 ml/fraction) were collected and analyzed for radioactivity using a scintillation counter. The purity of the labeled HS was determined by sensitivity to enzyme digestion with 5 milliunits of heparitinase I and II (Seikagaku Corp.) and heparinase (Seikagaku Corp.). Estimations of molecular mass values were derived from fractionation of several Dextran molecular size markers (Sigma) by gel chromatography and staining with orsinol solution.

Proliferation Assay—Two days after transfection, mES cells were harvested and replated in triplicate at 0.8×10^4 cells per well in 96-well 0.2% gelatin-coated plates in ES medium with LIF. Cell counting kit-8 (Dojindo) was added after 0 h, 24 h or 48 h and incubated further for 2 h. The soluble formazan product was measured at 450 nm.

Self-renewal Assay—Two days after transfection, mES cells were harvested and replated at 1×10^4 cells per gelatin coated 60 mm tissue culture dish in ES medium with LIF. For detection of undifferentiated cells, cells were fixed and stained with 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (Nacalai Tesque) 5 days after replating. Alkaline phosphatase (AP) positive colonies were counted by microscopic examination. Colonies of tightly packed and flattened AP-positive cells were counted as undifferentiated, and colonies of mixtures of unstained and stained cells and entirely unstained cells with flattened irregular morphology were considered differentiated.

Immunoblotting—Two days after transfection, the culture solution for the mES cells was replaced with serum-free ES medium without LIF for 4 h and the cells were stimulated for 20 min with one of the following: 15% FBS, 1000 units/ml LIF, 10 ng/ml BMP4 (R&D Systems), 40 ng/ml basic FGF (Upstate Biotechnology) or 15% FBS plus 1000 units/ml LIF. For depletion of HS chains, mES cells were incubated with 5 milliunits of heparitinase I and II and heparinase for 2 h before extrinsic stimulation. Cells were lysed with lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM Na_3VO_4, 10 mM NaF, protease inhibitors).

To isolate nuclear extracts, cells were first suspended in 100 μl of buffer (10 mM Hepes, pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, 10 mM KCl, 1 mM dithiothreitol, protease inhibitors). After incubation for 15 min on ice, 12.5 μl of 5% Nonidet P-40 was added, the suspension was vortexed for 10 s, and incubated for a further 5 min on ice. The suspension was centrifuged at 13,000 rpm for 30 s. The supernatant was removed and the pellets, comprising the nuclear extracts, were washed with PBS and lysed with lysis buffer (25 mM Hepes, pH 7.4, 500 mM NaCl, 1 mM dithiothreitol, 10% glycerol, 0.2% Nonidet P-40, 5 mM $MgCl_2$). The purity of the cell fractionation (cytosol and nucleus) was confirmed in Western blot analysis using an anti-Yes monoclonal antibody (BD Biosciences) and an anti-Lamin B_1 antibody (Zymed Laboratories Inc.); these antibodies are specific for the cytosol and nucleus, respectively. Only low levels of cross-contamination were observed (<1%).

Ten micrograms of cell lysates or nuclear extracts were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). After blocking, the membranes were incubated with antibodies to STAT3 (BD Biosciences), phosphorylated STAT3 (Tyr-705 BD Biosciences), ERK-1/2 (Cell Signaling Technology), phosphorylated ERK-1/2 (Thr-183 and Thr-185; Sigma), Akt (BD Biosciences), phosphorylated Akt (Ser-472 and Ser-473; BD Biosciences), phosphorylated Smad1 (Ser-463 and Ser-465; Cell Signaling Technology), Yes (Santa Cruz Biotechnology), phosphorylated Src family (Tyr-416; Cell Signaling Technology), β-actin (Sigma), β-catenin (Cell Signaling Technology), phosphorylated β-catenin (Ser-33/37/Thr41; Cell Signaling Technology), Lamin B_1, Oct3/4 (Santa Cruz Biotechnology), or Nanog (ReproCELL). The membranes were then incubated with the appropriate peroxidase-conjugated secondary antibody (Cell Signaling Technology). After washing, the membranes were developed with ECL Plus reagents (GE Healthcare). For detection of phosphorylated Yes, cells were lysed with lysis buffer (30 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, protease inhibitors) and immunoprecipitated with 1 μg of anti-Yes monoclonal antibody and protein G Magnetic Beads (New England Biolabs); this approach was adopted as this is the only commercially available anti-phosphorylated Src family antibody that cross-reacts with phosphorylated Yes.

Luciferase Reporter Assay and Immunostaining—Transactivation of β-catenin on T-cell-specific factor (Tcf) was determined with a luciferase reporter assay. siRNA expression plasmid (2 μg) was cotransfected with reporter plasmid such as, TOPFLASH (2 μg, containing three Tcf binding sites, Upstate Biotechnology) or FOPFLASH (2 μg, containing inactive Tcf binding sites, Upstate Biotechnology) and pCH110 (0.2 μg, containing β-galactosidase, GE Healthcare) as control of transfection efficiency using Lipofectamine 2000 as described above. Cell lysates were prepared 3 days after transfection and luciferase activity was measured with Dual-Light® System (Applied Biosystems). Luminescence was measured with a Lumat LB9501 luminometer (Berthold). Luciferase activity was normalized for transfection efficiency by β-galactosidase activity.