gested that synovial sarcoma cells penetrated the blood-nerve barrier, although the molecular mechanism of this transmigration across the vascular endothelium is obscure.

It is still unclear why the prevalence of metastases to peripheral nerves differs among tumours, most involve carcinoma, and there have been no previous reports of cases involving sarcoma. Modern neuro-imaging techniques have revealed that brain metastases occur in 20% to 35% of patients with primary carcinoma and in 5.6% of patients with sarcoma.[9,11] However, histological analysis has revealed a high incidence of metastasis to the brain in cases of sarcoma: in all of three angiosarcomas, three of four alveolar soft-tissue sarcomas and four of eight haemangiopericytomas, all of which had histologically epithelioid features.[11] This indirect clinicopathological evidence could indicate that neural metastasis through the blood-brain barrier or blood-nerve barrier is closely related to the expression of molecules associated with the maintenance of the epitheliod phenotype of the tumour cells.

Surgical exploration is sometimes the only way to arrive at a correct diagnosis of intraneural metastasis because the lesion may be too small to be detected by imaging methods.[3,7]

In a report of five cases, radiotherapy to the isolated metastasis in a brachial plexus provided good symptomatic relief to all patients and brought about local arrest of the disease in four of the five patients.[7] If an intraneural metastasis is diagnosed by MRI in a patient with a radial nerve palsy, radiotherapy might be an option for treatment. However, in the present case, because a precise diagnosis could not be made, excision was undertaken after obtaining informed consent. It may be that the lesion was too small to be detected by MRI scan. Segmental resection of the involved nerve, followed by nerve grafting if technically possible, may be an alternative option for obtaining relief from pain.

We have described the first published case of metastasis of a synovial sarcoma to a peripheral nerve. The patient had a progressive neural palsy with intractable neuralgia. The possibility of intraneural metastasis should be considered in a patient with a history of sarcoma who has a progressive nerve palsy and intractable pain without evidence of spinal metastasis. We recommend that in patients who have a peripheral neuropathy and a previously identified malignancy, surgical exploration should be considered.

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Tenascin-C levels in pseudosynovial fluid of loose hip prostheses

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Objective: Aseptic loosening is one of the most important problems that can occur after total hip arthroplasty (THA). In this study, we analysed levels of large tenascin-C (TN-C) variants and compared them in pseudosynovial fluid from patients with aseptic loosening after THA with those in synovial fluid from patients undergoing primary THA (control).
Methods: Pseudosynovial fluid samples (n=24) were obtained by aspiration at the time of revision THA performed due to aseptic loosening. Synovial fluid samples (n=12) were obtained by aspiration at the time of primary THA. Expression of TN-C splice variants was examined using immunoblotting. TN-C levels were measured using an enzyme-linked immunosorbent assay (ELISA) system that we developed previously.
Results: Western blotting showed the presence of large TN-C variants in pseudosynovial fluid of artificial joints with loosening. TN-C levels were approximately three times higher in pseudosynovial fluid of loose artificial joints (median 151.9 ng/mL) than in synovial fluid controls (median 50.1 ng/mL) (p=0.035).
Conclusion: Levels of TN-C including large variant subunits are elevated in pseudosynovial fluid of loose artificial joints, indicating that TN-C is a useful novel biochemical marker of loose hip prostheses.

Aseptic loosening is one of the most important problems that occur after total hip arthroplasty (THA). The causes of loosening are thought to be mechanical stress and biological reactions in the periprosthetic tissue against wear debris of ultra-high-molecular-weight polyethylene, metal, and bone cement (1). However, the precise biological mechanisms responsible for loosening remain unclear.

After THA, synovial membrane-like tissue forms in the artificial joint cavity and at the bone–implant or bone–cement interface. The principal cell types of this tissue are macrophages, fibroblasts, and giant cells with foreign body reaction. This synovial membrane-like tissue produces pseudosynovial fluid. Mazzucco et al (2) compared protein, phospholipid, and hyaluronic acid content between pseudosynovial fluid samples from hips with loosened arthroplasty and synovial fluid samples from osteoarthritic hips. Hyaluronic acid concentration was lower for the hips with loosened arthroplasty than for the osteoarthritic hips, whereas protein and phospholipid content did not differ between the groups. Analysis of pseudosynovial fluid could provide important information about the environment of synovial membrane-like tissue, and could indicate loosening after arthroplasty. Reports indicate that, in addition to laminin, vitronectin, and fibronectin (3, 4), high expression of tenascin-C (TN-C) has been detected in synovial membrane-like tissue of hips with loosening after THA (5).

TN-C is a hexameric glycoprotein component of the extracellular matrix (ECM). A subunit of TN-C includes heptad repeats, epidermal growth factor (EGF)-like repeats, fibronectin type III (FNIII)-like repeats, and a carboxyl-terminal globular domain shared with fibrinogens (6). The FNIII repeats between the conserved fifth and sixth repeats can undergo alternative splicing when the RNAs are processed. In adults, the small TN-C variant, in which the alternatively spliced repeats are spliced out, is weakly but constitutively expressed in static tissues (7, 8), whereas the large variants, containing the alternatively spliced FNIII domains in various combinations, are preferentially expressed in pathological tissues associated with regeneration, inflammation, and tumorigenesis (7–10).

Recently, we developed an enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody specific to the large splice variants of TN-C (11). The main purpose of the present cross-sectional study was to compare levels of large TN-C variants between

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pseudosynovial fluid from patients with aseptic loosening after THA and synovial fluid from patients undergoing primary THA.

Methods

Patients and samples

Pseudosynovial fluid samples (n=24; six men and 18 women) were obtained by aspiration at the time of revision THA performed due to aseptic loosening. The original THA was cementless in 19 hips and cemented in five hips. Component loosening was diagnosed by radiography, and was confirmed at the time of revision surgery. Radiographic loosening of the femoral component was assessed using the criteria of Harris et al (12) for cemented stems (subsidence or cement fracture) and the criteria of Engh et al (13) for cementless stems (progressive subsidence). Radiographic loosening of the acetabular component was assessed using the criteria of Hodgkinson et al (14) (migration or >1 mm of radiolucency in all zones). The median age of the 24 revision THA patients was 64.5 years (range 46–84 years), and their median body mass index (BMI) was 23.2 kg/m^2 (range 15.6–28.9 kg/m^2). The reason for the primary THA of the revision THA patients was osteoarthritis in 16 patients, idiopathic osteonecrosis of the femoral head in five patients, and trauma in three patients. The median time from primary THA to revision was 10.6 years (range 3.4–26.0 years). Osteolysis, which was defined using the method of Mulroy and Harris (15) (areas of endosteal, intracortical, or cancellous destruction of bone that were not linear, were >2 mm in width, and were or had been progressive), occurred in nine hips. No evidence of infection was observed during implant removal.

As controls, synovial fluid samples (n=12) were obtained by aspiration from patients undergoing primary THA (two men and 10 women). The preoperative diagnoses of the control group were osteoarthritis of the hip in eight patients and idiopathic osteonecrosis of the femoral head in four patients. As it would not be ethical to collect synovial fluid samples from patients with an asymptomatic stable hip, primary THA patients were selected as the best available control group. The median age of these control patients was 62.5 years (range 24–80 years), and their median BMI was 23.6 kg/m^2 (range 19.5–29.8 kg/m^2).

We found no differences between the two groups in terms of gender (p=0.691), age (p=0.400), or BMI (p=0.322). All patients gave their informed consent, and this study was approved by the local ethics committee. All patients had serum C-reactive protein concentrations within the normal range for healthy adults. Joint fluid was centrifuged at 15 000*g* for 15 min, and the supernatants were stored at −80°C until analysed.

Western blot analysis

Immunoblot analysis of pseudosynovial fluids was performed using a previously described method (11). Briefly, samples of eightfold diluted pseudosynovial fluids were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with a 2 to 15% polyacrylamide gradient. The electrophoresed proteins were blotted onto Immobilon membranes (Millipore Japan, Tokyo, Japan), blocked with blocking buffer, and incubated with monoclonal anti-TN-C antibody (4F10TT against the EGF-like domain, or 19C4MS against the FNIII C domain) at 4°C overnight; the characteristics of the antibodies used are described elsewhere (11, 16). After the membranes were washed in the blocking buffer, they were treated with peroxidase-labelled goat anti-mouse IgG Fab′ (1: 400, MBL, Nagoya, Japan) for 1 h at room temperature, followed by development in diaminobenzidine/H_2O_2 solution. The experiment was performed in duplicate.

ELISA

To quantify the levels of large-subunit TN-C, which contains the FNIII C domain, an ELISA was performed using an ELISA kit (IBL, Gunma, Japan) with two monoclonal antibodies: 4F10TT and 19C4MS (11). Samples were diluted 10-fold and incubated in 96-well ELISA plates coated with 19C4MS for 1 h at 37°C. After washing, horseradish peroxidase-conjugated anti-TN-C antigen-binding fragments (Fab′) (4F10TT Fab′) were added. After incubation for 30 min at 4°C, the absorbance at 450 nm was measured using an ELISA plate reader. Results were calculated using the mean absorbance of duplicate wells. TN-C purified from conditioned media of human glioma cells was used to prepare a standard curve. The detection limit of this assay was 0.4 ng/mL, and the intra-assay coefficient of variation was less than 10%.

Statistical analysis

The Mann–Whitney U-test and Fisher's exact test were used to detect differences between the groups. Correlation was estimated using Spearman's rank correlation test. The threshold of statistical significance was set at $p<0.05$.

Results

Western blot analysis

We assayed for the presence of large TN-C variants, which include the FNIII C domain, in pseudosynovial fluid of hips with loosening after THA. The antibody 4F10TT, which is specific to the EGF-like

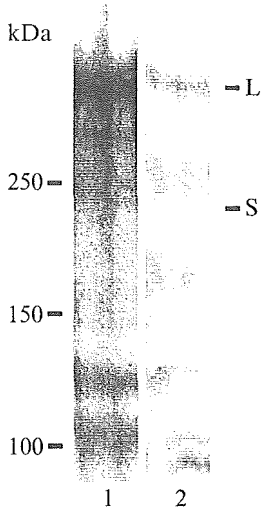

Figure 1. Representative Western blot analysis of pseudosynovial fluid using the anti-TN-C monoclonal antibodies 4F10TT (lane 1) and 19C4MS (lane 2). The positions of the largest (L) and smallest (S) bands of human glioma TN-C, which co-migrated on the gel, are indicated. In lanes 1 and 2, a major fraction of immunoreactive TN-C is detected at molecular weights corresponding to the L band. The degraded fragments of the large variants are detected below the L band. A small amount of the smallest variant is visible at S in lane 1. The pseudosynovial fluid of joints from loose hip prostheses contains a considerable amount of the large variants.

domain, reacted with all TN-C variants with molecular weights of 350 to 210 kDa in pseudosynovial fluid (Figure 1, lane 1). The main band at 350 kDa co-migrated with the largest variant of human glioma TN-C (L). The smallest variant (S), which lacks the alternatively spliced FNIII repeats and has a molecular weight of 210 kDa, was weakly labelled. Degraded fragments with molecular weights of around 100 kDa were labelled by 4F10TT. The antibody 19C4MS, which is specific for the large variants, reacted with the bands at 350 and 240 kDa, and also reacted with bands smaller than 200 kDa from the patient sample shown in lane 1. 19C4MS did not label the 210 kDa band (Figure 1, lane 2). These results indicate that the level of large TN-C variants is considerably elevated in pseudosynovial fluid of artificial joints with loosening.

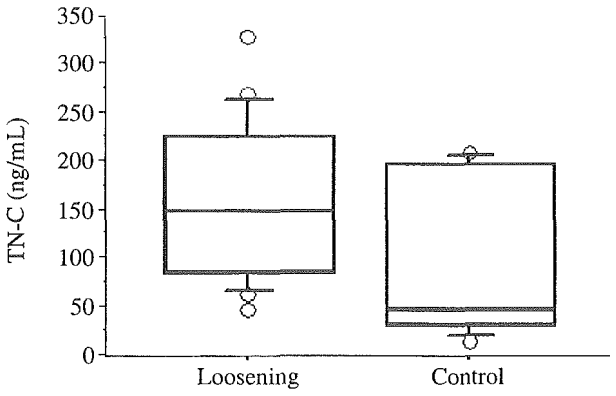

Figure 2. TN-C levels in loose artificial hip fluid and primary THA synovial fluid. Top, bottom, and middle lines of the box graph correspond to the 75th percentile, 25th percentile, and median, respectively. Bars indicate the range of the 10th and 90th percentiles. Each circle represents an outlier. Loosening: pseudosynovial fluid samples of loose artificial hip joints. Control: synovial fluid samples from patients undergoing primary THA.

ELISA

The TN-C levels in the pseudosynovial fluid of loose artificial joints (median 151.9 ng/mL) were approximately three times higher than those of the synovial fluid controls (median 50.1 ng/mL) ($p=0.035$) (Figure 2). In the pseudosynovial fluid of loose artificial joints, the TN-C levels were not significantly affected by gender ($p=0.689$), age ($p=0.335$), BMI ($p=0.166$), use of cement ($p=0.972$), osteolysis ($p=0.200$), or the interval from primary THA to revision ($p=0.953$).

Discussion

Aseptic loosening has been shown to be associated with chronic foreign body-type inflammation, which leads to activation of local inflammatory cells, particular cells of the monocyte/macrophage lineage. When periprosthetic macrophages are stimulated, their cytokine production is activated. Gait increases the pressure of pseudosynovial fluid in the artificial joint space, and the fluid can be forced along the interface between bone and prostheses, both on the femoral and acetabular sides, particularly if there is loosening (17). In addition, Schmalzried et al (18) proposed the concept of effective joint space, which includes all periprosthetic regions that are accessible to pseudosynovial fluid and thus accessible to particulate debris. Polyethylene wear is the major source of particulate wear debris. Polyethylene particles can migrate in the effective joint space far from the joint. When prosthetic wear debris is phagocytized by macrophages in synovial membrane-like tissue located near the component–bone interface, cellular activation occurs, which can cause osteolysis and loosening (1, 18). Pseudosynovial fluid from hips with loose prostheses also has a higher concentration of tumour necrosis factor-α (TNF-α) and interleukin-1 (IL-1) than synovial fluid from osteoarthritic hips (19, 20). Expression of cytokines, including TNF-α (21), IL-1 (21, 22), platelet-derived growth factor (23), basic fibroblast growth factor (24), and transforming growth factor β (25), is up-regulated in the periprosthetic tissue membrane of loose THA, compared with synovium of normal or osteoarthritic hips (26–29). Such changes in cytokine expression may induce an increase in local TN-C expression.

In addition, expression of TN-C in periprosthetic tissue may indicate the presence of a continuously ongoing wound-healing process. Increased micromotion of hip prostheses produces mechanical pressure

on the adjacent connective tissues. There is ample evidence from studies in which loads are applied or removed that TN-C expression is controlled partly by the level of mechanical stress (30, 31). Local production of TN-C in the effective joint space can be stimulated by soluble factors, as well as by mechanical stress (31), and can thus reflect the prosthetic loosening. In the early stage of prosthetic loosening, TN-C loosens adhesion of cells to ECM, and may help to minimize cellular damage caused by mechanical stress and promote tissue repair. However, high levels of TN-C may up-regulate expression of matrix metalloproteinases (MMPs). A combination of TN-C and fibronectin is reported to induce expression of MMP-1, -3, and -9 in synovial fibroblasts *in vitro* (32). MMP-1, -9, and -13 levels are also elevated in pseudosynovial fluid of loosened THA (33–35). These MMPs may promote loosening through degradation of ECM supporting the prosthesis. Thus, excessive tissue remodelling of the surrounding tissues could accelerate prosthetic loosening, which is associated with high TN-C expression.

Routine radiography is the most common technique used to visualize bone loss around the component interfaces after arthroplasty, but it is not sensitive for early detection of this condition, because usually no more than 50% of depletions of skeletal calcium are detectable by radiography (36). Findings of our previous study of osteoarthritic joints (11) suggest that biochemical markers are more sensitive than radiography for the detection of loosening. In the present study, TN-C levels were three times higher in pseudosynovial fluid of loose artificial joints than in synovial fluid of controls.

In conclusion, we have demonstrated that levels of large TN-C variants are elevated in pseudosynovial fluid of loose hip prostheses, indicating that these variants are useful biochemical markers of loosening after THA. Further investigation of the bidirectional function of TN-C in prosthetic loosening is needed to clarify the significance of these markers.

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Clinical Outcome of a Novel Photodynamic Therapy Technique Using Acridine Orange for Synovial Sarcomas¶

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ABSTRACT

Synovial sarcoma (SS) is one of common malignant soft-tissue tumors and is encountered most commonly in children and young adults. It frequently involves or invades major neurovascular structures and bones, and its local recurrence rate after simple resection has been reported to be as high as up to 80%. Because major nerves and vessels, as well as an adequate amount of bone, must be preserved to restore excellent limb function in cases of SS, a surgical technique entailing a low risk of local recurrence is needed. Based on the findings of recent experimental studies conducted by us using a mouse osteosarcoma model, we developed a novel therapeutic technique for SS, consisting of reduction surgery followed by photodynamic therapy using acridine orange (AO-PDT), with or without X-ray irradiation at 5 Gy. A preliminary study revealed that low-dose X-rays also excite AO like photons. After an initial study on cell cultures, this novel technique was applied to six cases of SS. A follow-up of the subjects to determine the clinical outcome revealed that none of the cases treated by AO-PDT, including the four cases treated by additional 5 Gy irradiation and the two cases not receiving any radiation, showed any evidence of recurrence or local/systemic complications during the follow-up period of 19–51 months after the surgery. Therefore, we believe that AO-PDT with 5 Gy irradiation may be an excellent novel therapeutic modality with reduction surgery to salvage excellent limb function in SS involving major nerves and vessels or bones.

INTRODUCTION

Recently, malignant bone and soft-tissue tumors have been treated with wide surgical resection of the tumor, sometimes followed by adjuvant therapies with radiotherapy or chemotherapy. In soft-tissue sarcomas, the local recurrence rate following surgery alone in the buttock, groin, thigh and areas below the knee has been reported to be 38, 13, 15 and 0%, respectively. While radiotherapy has been shown to be effective for local tumor control in some radiosensitive sarcomas, the effectiveness of chemotherapy in improving the prognosis has yet to be established for most soft-tissue sarcomas other than rhabdomyosarcomas and primitive neuroectodermal tumors (1).

Synovial sarcoma (SS) is one of the common malignant soft-tissue tumors and is encountered most frequently in children and young adults, the origin of which still remains unclear (1). The overall 5 year survival rates have been reported to range from 50 to 75% after surgery with or without adjuvant chemotherapy or radiotherapy. The efficacy of chemotherapy for SS is still under study. The local recurrence rate, which is one of the important prognostic factors in this cancer, has been reported to be as high as up to 80% after surgery with an inadequate surgical margin, without adjuvant radiotherapy (2–6). Therefore, one of the most important considerations in the treatment of this tumor is to ensure tumor resection with a clear, wide margin. On the other hand, in cases of deep-seated SS, which frequently involve or invade major nerves, vessels and bones, wide resection may cause severe dysfunction of the affected limb, and even amputation might be necessitated in such cases. To overcome these problems associated with surgery, we have recently developed a new limb salvage strategy involving intralesional or marginal tumor excision supported by photodynamic therapy (PDT) using acridine orange (AO), with or without low-dose irradiation with X-rays, which excite AO, just like photons (7–10). This study was undertaken to determine the efficacy and clinical outcome of AO-PDT in patients with SS of the extremities.

MATERIALS AND METHODS

In vitro study

Before being applied clinically, the effect of AO-PDT was investigated on human SS cell lines; two cell lines, namely, YaFuSS and SYO-1, were studied. The YaFuSS cell line, which was supplied by Toguchida of Kyoto University, was established from a monophasic-type SS obtained from a 27

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Abbreviations: AO, acriding orange; CDF, continuously disease free; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; IARC, International Agency for Research on Cancer; MRI, magnetic resonance imaging; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NED, no evidence of disease; PDT, photodynamic therapy; SS, synovial sarcoma.

Table 1. Patients' profile, tumor localization, stage and clinical outcome after AO-PDT

Case no.	Diagnosis	Location/depth	Age (in years)/sex	GTNM* stage	Follow-up period (in months)	Prognosis†	Recurrence
1	Synovial sarcoma	Wrist/surface	31/F	IIC	51	CDF	No
2	Recurrent synovial sarcoma	Knee/deep	22/M	IIB	44	NED	No
3	Synovial sarcoma	Thigh/deep	38/F	III	42	CDF	No
4	Synovial sarcoma	Thigh/deep	7/F	III	36	CDF	No
5	Synovial sarcoma	Thigh/deep	52/M	III	26	CDF	No
6	Synovial sarcoma	Knee/deep	11/M	IIB	19	CDF	No

*GTNM stage = American Joint Commission on Cancer GTNM Staging System of Soft Tissue.
†CDF = continuously disease free.

year old male and expressed the fusion gene SYT-SSX1 (J. Toguchida, personal communication). SYO-1, which was supplied by Ozaki of Okayama University, was established from a biphasic-type SS obtained from a 19 year old female and expressed the fusion gene SYT-SSX2 (11). Both cell lines were inoculated into 96-well plates (5×10^3) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37°C in a 5% CO_2 atmosphere. After 24 h incubation in a preconfluent cell growth condition, the medium of the wells was replaced with DMEM containing 0.01, 0.1 or 1.0 μg/ML of AO (Sigma-Aldrich C.,, St. Louis, MO). After 10 min of exposure to AO, the cells were illuminated with unfiltered light from a Xenon source (Sanei Electronics Co., Ltd., Osaka, Japan). For the control study, cells were cultured in AO-free or AO-containing medium but not exposed to light illumination, or cultured in AO-free medium followed by light illumination. The cell viability in each well was measured by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay at 30 min, and 3, 24, 48 and 72 h after exposure to light illumination, and the cell growth rate was determined in each group (n = 12).

Clinical study

From December 1999 to August 2002, six patients of SS who had no distant metastasis were recruited for this study. The patients comprised three males and three females, with an average age of 27 years (range, 7–52 years). In two of the six cases (cases 3, 4), the SS arose from the middle part of the thigh, very close to the femoral artery and vein; in one case, it arose from the inguinal region and invaded the femoral nerve, artery and vein; in two cases, it arose from the knee, close to the femur and the joint synovium; and in the remaining case, it arose from the wrist close to the ulnar nerve, artery and tendon of the flexor muscles. The tumor was classified as stage III in three cases (cases 3, 4, 5), stage IIB in two cases (cases 2, 6), and stage IIC in one case (case 1), in accordance with the American joint commission on cancer GTNM staging system of soft-tissue sarcomas. The follow-up duration of the patients ranged from 19 to 51 months (average, 36 months) (Table 1). All the patients, except for one, received two courses of chemotherapy preoperatively and 4 courses of chemotherapy with doxorubicin and ifosfamide postoperatively.

For cases 1, 2, 5 and 6, intralesional tumor excision, similar to conventional macroscopic curettage for benign soft-tissue tumors, was performed, and for cases 3 and 4, marginal resection of the tumor with partial curettage was performed (Table 1). These procedures were conducted with the aim of minimizing the extent of damage to intact muscles, bones, and major nerves and vessels in close contact or invaded by the tumor, in order to obtain good recovery of limb function after surgery.

In the next step, additional microscopic curettage with an ultrasonic surgical knife (Olympus Co. Ltd., Tokyo, Japan) was performed using a fluorescence surgical microscope; this procedure was conducted under fluorovisualization after local treatment of the tumor with 1 μg/mL of AO solution for 5 min, followed by washing out of the excess AO solution with saline and excitation with blue light, which most effectively excites AO to emit green fluorescence. The microscope was equipped with an interference filter (466.5 nm) for the selection of blue light from a Xenon lamp and an absorption filter (>520 nm) for observation of the green fluorescence of AO under a conventional surgical microscope (Carl Zeiss Co., Ltd, Oberkochen, Germany). After thorough microscopic curettage until complete disappearance of green fluorescence from the remnant tumor mass (Fig. 2), AO-PDT was applied by illumination of the tumor curettage area with blue light (5000 lx) for 10 min, again using the fluorescence surgical microscope. The surgical wound was then closed without washing out of the AO solution, and the resected area was immediately irradiated in four patients (cases 1, 4, 5, 6) with 5 Gy X-rays in the radiotherapy room, in order to provide the advantage of the strong cytocidal effect of AO excited by low-dose X-rays (Table 1). The AO concentration, light illumination time, lux level, which is fundamentally correlated with the light energy, and the radiation dose were determined from the data obtained from our preliminary studies in a mouse model (7–10). In particular, it might be mentioned that irradiation with 5 Gy X-rays was found to be sufficient to completely kill mouse osteosarcoma cells exposed to 1 μg/mL AO within 72 h (10).

This clinical trial was officially approved by the ethics committee of our university. Each patient and also a close family member gave their consent for the AO-PDT with or without 5 Gy irradiation after a full explanation of the method and purpose of the study (informed consent).

Before the AO-PDT, we investigated the sensitivity of the SS from each case (except case 5, for whom fresh material from the tumor could not be obtained) to AO by exposing fresh biopsy specimens *ex vivo* to 1 μg/mL of AO solution and observing them under a fluorescence stereoscope after illumination with blue light.

Local recurrence of the tumor was evaluated by magnetic resonance imaging (MRI) and thallium scintigraphy. Limb function after surgery was evaluated using the ISOLS criteria (12). Additionally, local and systemic complications induced by AO administration or AO-PDT with or without 5 Gy irradiation were evaluated based on the clinical symptoms and blood examinations.

RESULTS

In vitro study

The tumor cell growth of YaFuSS (Fig. 1) and SYO-1 (Fig. 2) cells cultured in medium containing AO at the concentration of 0.01, 0.1 or 1.0 μg/mL and exposed to blue light illumination was significantly inhibited at 24, 48 and 72 h as compared with that of

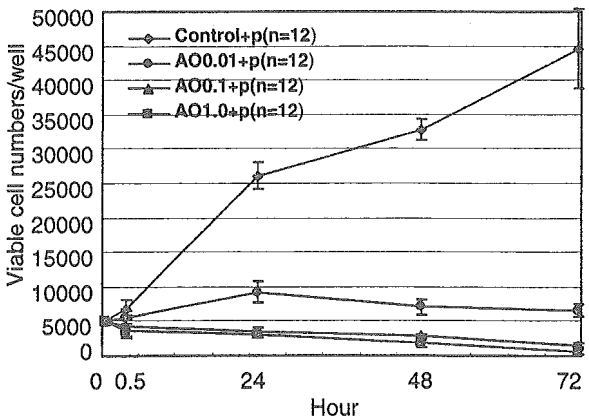

Figure 1. Tumor cell growth inhibitory effect of AO-PDT in the human SS cell line, YaFuSS (control+p: AO-free with light illumination, AO-0.01+p: 0.01 μg/mL AO with PDT, AO-0. 1+p: 0.01 μg/mL AO with PDT, AO-1.0+p: 0.01 μg/mL AO with PDT). Tumor cells exposed to 0.01, 0.1 or 1.0 μg/mL of AO, followed by light illumination died rapidly within 72 h.

control cells cultured in AO-free medium exposed or not exposed to blue light illumination or cells cultured in the presence of AO but not exposed to blue light illumination. The SYO-1 cells tended to be more sensitive to AO-PDT than YaFuSS cells. Cell death was indicated by cytoplasmic swelling, which was also observed in mouse osteosarcoma cells after treatment with AO-PDT (7).

Clinical study

All the tumor specimens studied, except for one, were determined to be sensitive to AO, based on their emission of green fluorescence after *ex vivo* exposure to AO solution and blue-light excitation.

Oncologically, all the patients enrolled in this study are alive (continuously disease-free [CDF], 5 patients; no evidence of disease [NED], one patient) at the time of writing, that is, April 2004, with no evidence of metastatic disease. No local recurrence of the tumor was detected by MRI or thallium scintigraphy in any of the patients who were followed up for 19 months or more (Table 1). The limb function in all the patients recovered to the level before the surgery and was therefore evaluated to be 100% by the ISOLS criteria (12). Clinically, none of the patients showed local or systemic complications related to AO administration or AO-PDT with or without 5 Gy irradiation.

DISCUSSION

AO, which was discovered in the 19th century as a weak basic dye, has been shown to have many unique biological activities, such as antitumor activity (13,14), photosensitizing activity (15,16) and toxicity against bacteria, malarial parasites and fungi (17–20). It has also been reported that AO has the capability of flowing rapidly into the cytoplasm through the cell membrane and binding to DNA (21), RNA (22) and lysosomes (23). However, our preliminary studies have revealed that AO binds mainly to RNA and not so avidly to DNA, to emit green fluorescence after blue light excitation in viable cultured mouse osteosarcoma cells, and also that it binds densely to lysosomes, to emit orange fluorescence (24). Because mouse osteosarcoma cells transplanted into the mouse emitted green fluorescence after intraperitoneal injection of AO followed by blue-light excitation while normal muscle and adipose tissue cells did not, the tumor could be visually localized under a fluorescence surgical microscope (fluorovisualization effect) (9). Although the mechanism underlying the selective binding of AO to musculoskeletal sarcomas is not clear yet, AO staining has nonetheless been shown to be useful for visual localization of the SS during surgery under a fluorescence microscope. We also found that AO exerted a strong cytocidal effect against mouse osteosarcoma cells after blue-light illumination, both *in vitro* (7) and *in vivo* (8). Based on these findings, we considered that AO might be useful for photodynamic therapy of musculoskeletal sarcomas. Many experimental studies have previously reported that AO has photosensitizing properties and is useful for photodynamic therapy of cancer (25–29); however, there are as yet no reports of the clinical application of AO in cancer therapy. While the reasons for this are not clearly understood, it is likely that investigators are wary of the potential toxic effects of AO, *e.g.* AO has been reported to exert mutagenic activity in bacteria (17,18). Nonetheless, the carcinogenicity of AO has never yet been experimentally proven (30). An International Agency for Research on Cancer (IARC) report (31) in 1978 classified AO as a group 3 agent, which means that the agent is not classifiable as to its carcinogenicity in humans. Local administration of AO for clinical gastric and cervical cancer screening has been reported (32), but none of the subjects has developed new cancer induced by AO. Although photodynamic therapy using porphyrin or its derivatives, which are commonly used for some cancers (33), commonly involves the use of a laser beam, which is high energy focused over a narrow area, as the excitation light source, we used a high-power Xenon lamp because illumination of blue light over a wide area is necessary for the fluorovisualization effect of AO as well as for the strong cytocidal effect of AO-PDT on remnant tumor cells, which are dispersed widely throughout the surgical field by curettage.

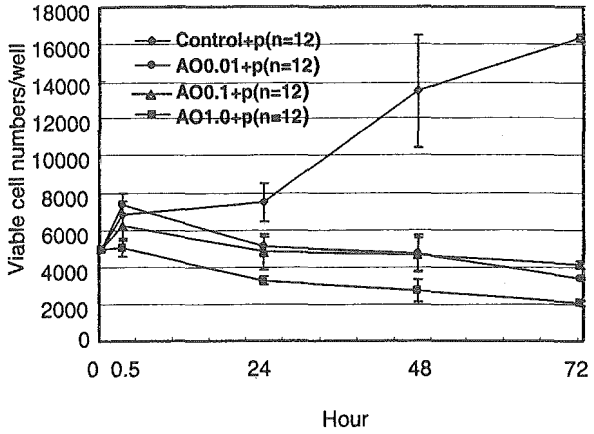

Figure 2. Tumor cell growth inhibitory effect of AO-PDT in the human SS cell line, SOY-1 (control+p: AO-free with light illumination, AO-0.01+p: 0.01 μg/mL AO with PDT, AO -0. 1+p: 0.01 μg/mL AO with PDT, AO-1.0+p: 0.01 μg/mL AO with PDT). Tumor cells exposed to 0.01, 0.1 or 1.0 μg/mL of AO, followed by light illumination died more rapidly within 72 h than the YaFuss cells.

Before clinical application of AO-PDT to human sarcomas, we performed a simulation study of curettage supported by AO-PDT in a mouse model (8). The results showed that AO-PDT significantly inhibited local tumor recurrence after macroscopic and microscopic curettage of a mouse osteosarcoma. While the recurrence rate was 80% in the control group, it was 23% in the group receiving AO-PDT. Furthermore, we also found that low-dose X-ray irradiation with 5 Gy of a mouse osteosarcoma exposed to AO showed the same strong cytocidal effect as that of AO-PDT (10). X-ray irradiation has the advantage of reaching deeper areas of the human body than a light beam, even though it is more injurious to normal tissues. AO also invades deeper tissues quickly at the rate of 1 cm/h (unpublished data). These results of our preliminary studies suggest that AO-PDT with or without 5 Gy irradiation might be applicable for limb salvage in cases of malignant bone and soft-tissue tumors. In cases where it is effective, almost full recovery of normal limb function can be expected, with only a low risk of local recurrence.

SS is one of the common malignant soft-tissue tumors that is most frequently encountered in children and young adults. The effectiveness of chemotherapy in SS remains to be clarified, although many retrospective studies have suggested that chemotherapy with ifosfamide and doxorubicin might be effective (2–6). Surgical resection is, therefore, mandatory for local control. After simple tumor excision, the local tumor recurrence rate is considerably high (60~80%). Even after wide resection, local recurrence occurs at a frequency of 5~30%. SS frequently invade or are in close contact with major nerves or vessels. The tumor in four of the six cases in our present study was in close contact with major nerves

and vessels; in one case, it was in close contact with the bony cortex; and in one, it was in contact with the joint capsule. Wide resection in these cases would also entail sacrifice of nerves, vessels and muscles, resulting in poor limb function. To ensure adequate recovery of limb function, it would be essential to preserve major nerves and vessels by reduction surgery using a marginal or intralesional procedure; however, this would result in a tumor-positive margin and entail the risk of local tumor recurrence. Therefore, some adjuvant therapy is essential to inhibit local tumor recurrence. It has been reported that postoperative radiotherapy is effective for local control for resected SS with a tumor-positive margin (4). However, radiation does not completely inhibit local recurrence in margin-positive cases, and radiation may induce limb dysfunction at a later stage with complications, such as joint contracture, skin sclerosis, subcutaneous or muscular fibrosis.

Logically, photodynamic therapy using acridine orange (AO-PDT) followed by 5 Gy irradiation should be able to kill the AO-binding tumor cells quickly and completely without any complications. An *in vitro* study revealed the sensitivity of two cell lines of human SS to AO, and AO-PDT showed a strong cytocidal effect on these cells expressing SYT-SSX 1 and SYT-SSX 2, respectively. In a clinical study, biopsy specimens obtained from five of the six tumors were confirmed to be sensitive to AO, because they emitted green fluorescence after *ex vivo* exposure to AO under a fluorescence stereoscope.

Examination of the clinical outcome of AO-PDT revealed that none of the cases treated by AO-PDT with (four cases) or without (two cases) 5 Gy irradiation showed any evidence of local recurrence during the follow-up period of 19–51 months after surgery. Because all of the six cases showed a histologically positive tumor margin and did not receive the conventional postoperative radiation with 50–60 Gy, the local recurrence rate in these cases of SS would have been expected to be more than 70% (2–6). Generally, in 80% of cases of malignant soft-tissue tumors, local recurrence occurs within 2 years after surgery (1). Therefore, we concluded that AO-PDT with or without 5 Gy irradiation may be useful for local control after intralesional tumor excision in patients of SS. There were also no systemic or local complications after AO-PDT, while photodynamic therapy with porphyrin or its precursors frequently causes the serious complication of light-sensitive dermatitis.

In conclusion, based on the results obtained from *in vitro* and clinical studies, we believe that AO-PDT with 5 Gy irradiation may be an effective novel adjuvant therapeutic modality to reduction surgery for SS involving major vessels, nerves or bones, to allow excellent recovery of limb function in these cases.

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