decrease in retinoblastoma (Rb) protein phosphorylation [88]. PKC-ε activated Akt in order to mediate anti-apoptotic functions [89]. PKC-η inhibited tumor necrosis factor-α (TNF-α)-induced cell death by blocking caspase activation [65], and was shown to be associated with the cyclin E/cyclin-dependent kinase 2 (Cdk2) complex [90]. aPKC-ζ blocked the phosphorylation of Akt [91], and was shown to be involved in EGF-stimulated chemotactic signaling in breast cancer cells [92]. The levels of PKC activity were reported to be significantly higher in tumor tissue from breast cancer patients than in normal tissue [93]. Changes in the expression and localization of PKC-η [94], and the down-regulation of PKC-α in clinical samples from breast cancer patients [95], have also been reported. Each PKC isozyme might thus have different functions affecting the proliferation, differentiation, and death of breast cancer cells, and these might vary in different cell types.

2.1.3. Effect of PKC in Hormone Therapy

Hormone therapy is an important treatment for breast cancer, and PKC might also be associated with hormone dependence in breast cancer cells. The anti-estrogen agent tamoxifen (TAM) has been reported to induce PKC-ε translocation to the cell membrane and to inhibit growth [96]. The over-expression of PKC-α regulated the estrogen receptor (ER) [97], and elevated PKC-α expression might predict resistance to TAM [98, 99]. PKC-δ has also been shown to play a major role in anti-estrogen resistance in breast tumor cells [100]. These results suggest that PKC might regulate responses to hormone therapy.

2.1.4. Relationship Between PKC and MAPK in Breast Cancer

Although the downstream events following PKC activation have not yet been fully clarified, the MAPK pathway is known to play a major role [101–103]. The relationship between PKC and MAPK has been studied in breast cancer by several investigators. HER2 and PKC-δ have been shown to be involved in the activation of ERK MAPK by estrogen [104], and PKC-α, PKC-β, and PKC-δ translocation has been shown to precede the acceleration of ERK MAPK phosphorylation and cell proliferation, as induced by angiotensin II [105]. Recently, Lee *et al.* [106] reported that the nucleoside analog sangivamycin induced apoptotic cell death in MCF7/adriamycin-resistant cells *via* PKC-δ and c-jun NH_2-terminal kinase (JNK) activation. We investigated the effects of activating endogenous PKC on cell proliferation and the cell-cycle by treating the breast cancer cell line SKBR-3 with PMA. PKC induced cell-cycle arrest in the G_1 phase in the SKBR-3 breast cancer cell line by a mechanism involving a PKC–ERK MAPK–JNK–Rb protein signaling pathway [107]. Other reports have implicated PKC in cell-cycle regulation, and have demonstrated a relationship between PKC and MAPK signaling [108–111]. Together with our data, these findings support the notion that PKC is an important cell-cycle regulator, through the PKC–MAPK pathway. If the apparently close relationship between PKC and MAPK could be clarified in breast cancer cells, both enzymes might be novel targets for breast cancer therapy. The reported roles of the various PKC isozymes in breast cancer are summarized in Table **2**.

2.1.5. PKC Inhibitors

As the intracellular signaling pathway involving PKC plays an important role in tumor growth, known PKC inhibitors have been investigated in both preclinical and clinical studies, with the aim of identifying potential selective anticancer drugs that do not have cytotoxic side effects.

Staurosporine and its analog UCN-01 are potent PKC inhibitors that have been shown to have strong antiproliferative activity, as assessed by the modulation of PKC substrate phosphorylation and the DNA damage-related G_2 checkpoint [112]. Preliminary data from clinical trials of UCN-01 indicated anti-tumor activity in melanoma and lymphoma patients, and the drug was reasonably well tolerated in terms of toxicity [112].

Bryostatin is an anti-neoplastic agent that interacts with both the cPKC and the nPKC subfamilies, and, in the presence of phorbol ester, acts as an antagonist [113]. In clinical trials, anti-tumor activity has been demonstrated for Bryostatin-1 in patients with melanoma, ovarian cancer, lymphoma, and leukemia [114–116].

The staurosporine analog PKC412 is an oral inhibitor of cPKC and nPKC, and has been shown to be an effective antiproliferative agent against various tumors in preclinical studies [117, 118]. Interestingly, inhibiting cPKC with PKC412 led to the inactivation of MAPK and affected c-fos expression *in vitro* [119]. Moreover PKC412 down-regulated ERK2 expression in cancer patients [120]. Clinical trials showed that PKC412 was not remarkably toxic and could be safely administered as a long-term oral drug [121, 122]. Phase I studies of PKC412 in combination with gemcitabine and cisplatin or 5-fluorouracil have also been carried out in cancer patients [123, 124].

The antisense phosphorothioate oligonucleotide ISIS3521 has been shown to selectively inhibit PKC-α expression and to have anti-tumor activity [125]. Phase I and II clinical trials of this drug in various cancer patients [126–129] have demonstrated anti-tumor effects in lymphoma, and, in combination therapy with cisplatin and gemcitabine, anti-tumor effects have been reported in patients with advanced non-small cell lung cancer [130–132].

The specificity trials and clinical trials that have been carried out on PKC inhibitors are summarized in Table **3**. Although several clinical trials have investigated PKC inhibitors in patients with

Table 2. Role of PKC in Breast Cancer Cells

Isozyme	Function	References
PKC-α	Involved in ER expression and TAM resistance Related to cell proliferation Regulated transcription from MDR promoter	[97–99] [85] [86]
PKC-β	Enhanced cell growth	[87]
PKC-δ	Involved in anti-estrogen resistance Blocked cell proliferation Involved in cell cycle	[100] [88] [107]
PKC-ε	Activated Akt to protect from cell death Translocated with TAM	[89] [96]
PKC-η	Involved in cell cycle Inhibited cell death	[90] [65]
PKC-θ	Regulated transcription from MDR promote	[86]
PKC-ζ	Related to chemotaxis Blocked the phosphorylation of Akt	[92] [91]

Abbreviations: ER, estrogen receptor; TAM, tamoxifen; MDR, multidrug resistance.

Table 3. PKC Inhibitors

Drugs	Specificity	Clinical trials	Reference
Staurosporine	Modulation of PKC substrate phosphorylation	Melanoma, lymphoma	[112]
UCN-01	DNA damage-related G_2 checkpoint		
Bryostatin	Interacts with cPKC and nPKC	Melanoma, ovarian cancer lymphoma, leukemia	[113–116]
PKC412	Inhibitor of cPKC and nPKC	Lung cancer, breast cancer gall bladder carcinoma	[117–124]
ISIS3521	Antisense phosphorothioate oligonucleotide Inhibit PKC-α expression	Lymphoma, lung cancer	[125–132]

malignant tumors, the effects have been unclear. One limitation of the current PKC inhibitors is that they are relatively non-specific in their actions, and we cannot yet fully exploit their potential for the differential inhibition of diverse PKC functions. Further translational research is needed to establish which PKC inhibitors will be most effective, and which combination therapies (with drugs such as trastuzumab for example) should be tested for breast cancer patients.

2.2. Cap43/NDRG1/Drg-1: A Putative Metastasis Suppressor Gene

2.2.1. Role of Cap43/NDRG1/Drg-1 in Human Cancers

Cap43, also known as N-myc downstream-regulated gene 1 (NDRG1), the human homocysteine-inducible gene [133] and differentiation-related gene-1 (Drg-1) [134], encodes a 43-kD protein, the expression of which is induced by nickel and calcium [135]. The Cap43 protein has three unique 10 amino-acid tandem-repeat sequences at its carboxyl terminus and is phosphorylated by protein kinase A (PKA) [136]. Cap43 expression is reduced in tumor cells [137], and is markedly affected by many stimuli including oxidative stress, metal ions, hypoxia, phorbol esters, vitamins A and D, steroids, histone deacetylase-targeting drugs, β-mercaptoethanol, homocysteine, and tunicamycin, as well as the oncogenes N-myc and C-myc, and the tumor-suppressor genes p53 and von Hippel–Lindau (VHL) [133, 135, 137, 138–142].

Although a number of studies have described its characteristics, the function of the Cap43 protein remains unclear. Cap43 is expressed in various organs including the prostate, ovary, colon, and kidney, and its expression patterns change dynamically during postnatal development in the kidney, brain, liver, and nerves [133, 143–145]. These observations suggest that Cap43 might be involved in organ maturation and differentiation.

Mutations in Cap43 were originally shown to be responsible for Charcot–Marie–Tooth disease type 4D, which is a hereditary neuropathy of the motor and sensory systems. Okuda *et al.* [146] recently established Cap43 knockout mice that exhibited Schwann cell dysfunction, suggesting that Cap43 is essential for the maintenance of the myelin sheaths in the peripheral nerves. Consistent with these findings, Hirata *et al.* [147] reported that Cap43 played an important role in the terminal differential of Schwann cells during nerve regeneration. Thus, Cap43 appears to have an important function in the development of the nervous system.

In contrast to these studies, Stein *et al.* [148] showed the Cap43 gene to be up-regulated by p53 and to be required for p53-dependent apoptosis, indicating that it is a p53 target gene. Furthermore, Kim *et al.* [149] reported that the Cap43 protein was associated with microtubules in the centrosome and participated in the spindle checkpoint in a p53-dependent manner, suggesting that it might play a key role in the regulation of microtubule dynamics. Over-expression of the Cap43 gene has also been reported to inhibit growth in colon cancers, as well as metastasis in prostate and breast cancer cells [150–152], suggesting that it suppresses metastasis. Cap43 expression was found to be increased in many types of human tumor, including colon, breast, prostate, kidney, liver, and brain cancers, compared with normal tissue [153]. However, Cap43 expression was reported to be up-regulated in normal cells and highly-differentiated cancer cells, but down-regulated in poorly differentiated cancer cells in colon and prostate tumors [150, 151]. Low levels of Cap43 expression in breast cancer cells were closely correlated with poor clinical outcomes [154]. Cap43 expression thus appears to be closely associated with the differentiation and/or malignant states of a variety of human cancers.

Recently, Maruyama *et al.* [155] reported a relationship between Cap43 and tumor growth and angiogenesis in pancreatic cancer. The over-expression of Cap43 in pancreatic cancer cells *in vivo* resulted in a marked decrease in growth rates and tumor-induced angiogenesis. In addition, the gelatinolytic activity due to matrix metalloproteinase-9 was markedly decreased in pancreatic cancer cell lines expressing high Cap43 levels, as was the cells ability to invade Matrigel. Immunohistochemical analyses of pancreatic ductal carcinomas showed a significant association between Cap43 expression and the density and depth of invasion of microvessels in the tumors, the histopathological grading, and the overall survival rate of patients. These results strongly suggest a key role for Cap43 in the control of the angiogenetic on–off switch in the tumor stroma in pancreatic ductal carcinoma.

2.2.2. Estrogen Induces Down-Regulation of Cap43 in Breast Cancer

As previous studies had suggested that the Cap43 gene might play a key role in breast cancer, we examined how its expression was modulated during therapeutic treatment with anti-estrogenic drugs, in order to assess its potential as a molecular indicator of the effects of anti-estrogenic agents in breast cancer. Among the eight breast cancer cell lines that we examined, four expressed high levels of Cap43 and very low levels of ER-α, whereas the other four expressed low levels of Cap43 and high ER-α levels. Treatment with estradiol (E_2) reduced Cap43 expression in a dose-dependent fashion in ER-α-positive cell lines, but had no effect in ER-α-negative lines. Administration of the anti-estrogens TAM and ICI 182780 abrogated the E_2-induced down-regulation of Cap43. Over-expression of ER-α in the ER-α-negative cell lines SKBR-3 and MDA-MB-231 resulted in the down-regulation of Cap43. Immunostaining studies showed that Cap43 expression was inversely correlated with the expression of ER-α. The E_2-induced down-regulation of Cap43 appeared to be mediated through ER-α-dependent pathways in breast cancer cells, both in culture and in patients [156]. As the expression of Cap43 is sensitive to modulation by E_2 and/or anti-estrogens in ER-α-positive breast cancer cells, it is a potential molecular indicator of the therapeutic efficacy of anti-estrogenic agents in breast cancer, as illustrated in Fig. (**5**). Although it is relatively easy to measure the amount of Cap43 in the blood after an operation, Cap43 is not a secrete protein so that Cap 43 should not appear in the blood. To address this clinical

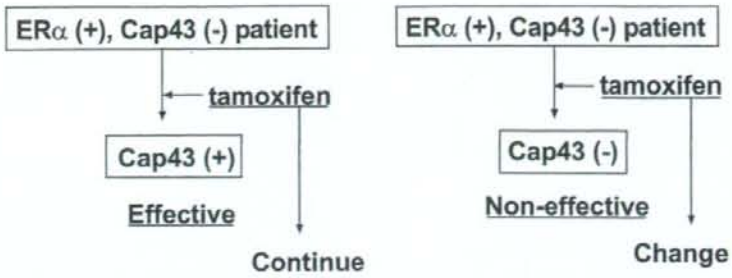

Fig. (5). Model of Cap43 application in breast cancer therapy. When ERα(+), Cap43(−) patients are treated with TAM and Cap43 becomes up-regulated, TAI seems to be effective, and the treatment should be continued. By contrast, when ERα(+), Cap43(−) patients are treated with TAM and Cap43 is not up regulated, TAM seems to be ineffective, and the treatment should be discontinued.

problem, we are now searching for a protein in the blood that is associated with Cap43. By measuring the level of such a protein, we might be able to indirectly determine the level of Cap43. Further study is needed before Cap43 can be used in clinical trials.

2.3. Y-Box Binding Protein-1 (YB-1)

2.3.1. Role of YB-1 in Human Cancers

YB-1 is a member of the cold-shock domain protein family. It has pleiotropic functions in the regulation of gene transcription and translation, DNA repair, drug resistance, and cellular responses to environmental stimuli [157–159]. YB-1 is normally present in the cytoplasm of human cells, but is translocated to the nucleus when they are exposed to anticancer drugs or ultraviolet (UV) light [160, 161]. This process is necessary in order for YB-1 to effectively control both the transcription of MDR-related genes and the repair of DNA damage induced by anticancer agents or radiation, resul ing in the acquisition of global drug resistance to a wide range anticancer agents [158, 162]. The nuclear translocation of YB-1 controlled by PKC and related proteins, protein tyrosine phosph tase, JAK1, and Akt [161, 163–167]. In immunohistochemica labeling experiments, nuclear YB-1 expression was correlated wi the expression of a representative MDR-related ATP-binding ca sette superfamily protein, P-glycoprotein, encoded by t MDR1/ABCB1 gene, and other drug resistance-related molecul have been reported in a variety of tumors in addition to breast ca cer [168–173]. By contrast, nuclear expression of YB-1 is oft associated with poor prognosis in various human malignancie including breast cancer [168, 170], ovarian cancer [172], synovi sarcoma [174], and lung cancer [175]. These findings strongly su gest that the nuclear expression of YB-1 might have a predicti value in some human malignancies. Furthermore, Bergmann *et a*

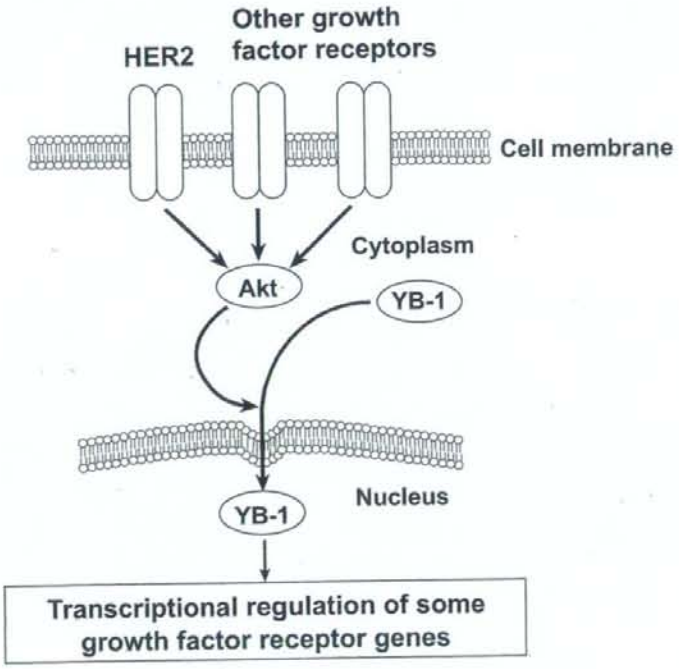

Fig. (6). Association between the expression of nuclear YB-1 and HER2. The nuclear expression of YB-1 is thought to play an essential role in the acquisitic of some malignant characteristics, as a result of the activation of HER2–Akt dependent pathways, in breast cancer patients.

[176] reported that introducing the YB-1 gene could induce breast cancer in experimental animal models, implying that it is an oncogene that promotes the progression of breast cancer. Berquin *et al.* [177] reported that YB-1 over-expression in human mammary epithelial cells could induce an EGF-independent growth phenotype through activation of the EGFR pathway. Together, these studies point to a close association between YB-1 expression and the growth or proliferation potential of cancer cells, which might affect the prognosis of breast cancer patients.

2.3.2. Role of Nuclear YB-1 Localization In Breast Cancer

As YB-1 could control the expression of both drug resistance-related genes and cell growth-related genes, we investigated the relationship between YB-1 localization in the nucleus and the expression of EGFR family proteins, hormone receptors, and other molecules that might be associated with poor prognosis in breast cancer patients. We found that nuclear YB-1 expression was correlated with HER2 expression, but not EGFR expression, in clinical human breast cancer specimens (Fig. **6**). Immunostaining studies showed that nuclear YB-1 expression was an independent prognostic indicator of overall survival (Fujii *et al.* unpublished data). Nuclear YB-1 localization therefore seems to be an important molecular target not only in the acquisition of MDR, but also in tumor growth dependent upon HER2 and other growth factor receptors in breast cancer.

3. CONCLUSION

The development of effective adjuvant therapies, based on the post-operative administration of chemotherapy, hormone therapy, or trastuzumab, has significantly improved survival for breast cancer patients [178]. However, the treatment of adjuvant-resistant or metastatic disease is still palliative, and there is a low probability of inducing complete remission or discovering a definitive cure for breast cancer. Research efforts in this area of basic research have identified key selective changes, in molecules such as PKC, Cap43, and YB-1, which sustain breast cancer growth and progression. These findings present opportunities for developing specific targeted therapies, which hold great promise as the next generation of anticancer therapeutics.

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CONFLICT OF INTEREST

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Different expression profiles of Y-box-binding protein-1 and multidrug resistance-associated proteins between alveolar and embryonal rhabdomyosarcoma

Yoshinao Oda,[1,6] Kenichi Kohashi,[1] Hidetaka Yamamoto,[1] Sadafumi Tamiya,[1] Kimitoshi Kohno,[4] Michihiko Kuwano,[5] Yukihide Iwamoto,[3] Tatsuro Tajiri,[2] Tomoaki Taguchi[2] and Masazumi Tsuneyoshi[1]

[1]Department of Anatomic Pathology, [2]Pediatric Surgery, and [3]Orthopedic Surgery, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582; [4]Department of Molecular Biology, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555; [5]Research Center for Innovate Cancer Therapy, Kurume University, 67 Asahi-machi, Kurume 830-0011, Japan

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Nuclear expression of the Y-box-binding protein-1 (YB-1) has been reported to regulate the expression of both P-glycoprotein (P-gp) and major vault protein (MVP), and to regulate proliferative activities in human malignancies. Based on morphology and molecular biology, rhabdomyosarcoma (RMS) can be divided into two major types: embryonal type and the more aggressive alveolar type. Thirty-five cases of embryonal RMS (ERMS) and 28 cases of alveolar RMS (ARMS) were examined immunohistochemically for the nuclear expression of YB-1 and the intrinsic expression of P-gp, multidrug resistance (MDR)-associated protein (MRP) 1, 2, and 3, breast-cancer resistant protein (BCRP) and MVP, and the findings were compared with proliferative activities as evaluated by the MIB-1-labeling index (LI). Moreover, mRNA levels of these MDR-related molecules were assessed using a quantitative reverse transcriptase-PCR method in 18 concordant frozen materials. P-gp expression was more frequently observed ARMS, compared with ERMS ($P = 0.0332$), whereas immunoreactivity for BCRP was more frequently recognized in ERMS ($P = 0.0184$). Nuclear expression of YB-1 protein was correlated with P-gp ($P = 0.0359$) and MVP ($P = 0.0044$) expression, and a higher MIB-1-labeling index ($P = 0.0244$) in ERMS, however, in ARMS no such relationships were observed. These immunohistochemical results indicate that different expression profiles of MDR-related molecules and their correlation with YB-1 nuclear expression support the concept that ERMS and ARMS are molecular biologically distinct neoplasms. Apart from ERMS, frequent P-gp expression in ARMS may be independent from YB-1 regulation. However, YB-1 may be a candidate for a molecular target in rhabdomyosarcoma therapy, especially in ERMS. (*Cancer Sci* 2008; 99: 726–732)

Y-box-binding protein-1 (YB-1) has been reported to be as a transcription factor which interacts with the inverted CCAAT-box (Y-box) in promoters and enhancers of multiple genes. YB-1 has been reported to play a critical role in cell proliferation, DNA replication and drug resistance.[1,2] In particular, previous studies have implicated YB-1 as a regulatory factor for the multidrug resistance (MDR)1 gene in human malignancy.[1,2] MDR is a frequent cause of treatment failure in cancer patients. One mechanism of MDR is overexpression of ATP-binding cassette (ABC) transporter proteins that function as a drug efflux pump. These ABC transporter proteins include MDR1/P-glycoprotein (P-gp),[3] a number of the multidrug resistance-associated protein (MRP) family,[4] and the recently identified breast cancer resistance protein (BCRP).[5] The lung resistance-related vault protein (LRP) has been identified as the major vault protein (MVP), which is also associated with MDR.[6]

Nuclear expression of YB-1 has been reported to have a close relationship with MDR1/P-gp expression,[7–9] or poor prognosis in several human malignancies.[7,9] YB-1 also has been reported to promote basal and 5-fluorouracil-induced expression of the *MVP* gene, the promoter of which contains the Y-box in human colon cancer.[10] Moreover, nuclear expression of YB-1 has demonstrated significant correlation with intrinsic MVP expression in ovarian cancer.[11]

Rhabdomyosarcoma (RMS) is the most common malignant soft tissue neoplasm in children.[12] Based on histopathological features, RMS can be categorized into two major types: embryonal and alveolar subtype.[13] Alveolar RMS (ARMS) emerges as morphologically, genetically and biologically distinct from embryonal RMS (ERMS).[14–16] ARMS harbors non-random chromosomal translocations t(2;13)(q35;q14) or t(1;13)(p36;q14) that lead to the fusion of *PAX3* and *PAX7*, respectively, to *FKHR*.[17,18] In ERMS no diagnostic specific genetic alterations have been demonstrated, however, molecular analysis of polymorphic loci revealed allelic loss in chromosomal region 11p15 in most cases.[19,20] RMS is now commonly treated using chemotherapeutic agents,[21,22] including vinca alkaloids, anthracyclines, etoposide, cyclophosphamide, and ifosfamide, which are involved in the substrates of the above ABC transporters,[3–5] or MVP.[6]

In this study, we analyzed the correlation between YB-1 nuclear expression and the intrinsic ABC transporter or MVP expression in 63 cases of RMS and the results of these expression patterns were compared between ARMS and ERMS. Furthermore, we compared intrinsic ABC transporter or *MVP* mRNA expressions between 18 cases of RMS and six samples of normal skeletal muscle, using the real-time quantitative reverse transcriptase (RT)-PCR method.

Materials and Methods

Case materials. Sixty-three cases of primary rhabdomyosarcoma registered in the soft-tissue sarcoma files at the Department of Anatomic Pathology, Kyushu University between 1971 and 2004 were available for immunohistochemical study. The diagnosis of all cases was based on light microscopic examination with hematoxylin-eosin staining according to the World Health Organization classification in 2002,[13] and, where necessary, immunohistochemical analysis was carried out

[6]To whom correspondence should be addressed.
E-mail: oda@surgpath.med.kyushu-u.ac.jp

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including myogenin and/or MyoD1 expression to confirm skeletal muscle differentiation. All the samples were collected prior to the administration of any therapy. Among them, frozen material was available in 18 cases for the evaluation of mRNA expression. In all seven cases of ARMS where frozen material was available, PAX3-FKHR transcripts was detected by RT-PCR. Furthermore, six frozen samples of normal skeletal muscle from patients with other diseases were used as a non-tumorous control. Survival data were available for 61 cases. Follow up ranged from 2 to 223 months (mean, 47 months). Patients were considered as adults when diagnosed at 16 years or older.[23] The sites of the primary tumor were divided into favorable site (orbit, head and neck [excluding parameningeal]) and genitourinary system (non-bladder/non-prostate) and unfavorable site (bladder/prostate, extremity, parameningeal head and neck, and other sites [including trunk, retroperitoneum etc.]). Forty-two patients were treated with chemotherapy and surgery, eight were treated with surgery and irradiation, and six were treated with surgery, chemotherapy and irradiation. Three patients were treated with surgery alone, but details of the therapy were unknown in four patients. Before the treatment, the disease was classified into four stages according to the TNM staging system developed by the Intergroup Rhabdomyosarcoma Study (IRS).[24]

Immunohistochemistry. Formalin-fixed, paraffin-embedded blocks containing the most viable parts of the tumor were selected in each case. The following monoclonal antibodies were used as primary antibodies: anti-P-gp (JSB-1; 1:20; Sanbio, Uden, Netherlands), anti-MRP1 (MRPr1; 1:50; Nichirei, Tokyo, Japan), anti-MRP2/cMOAT (M_2III-6; 1:20; Sanbio), anti-MRP3 (M3 III-6, 1:80; Kamiya Biomedical Company, Seattle, WA, USA), anti-MVP (LRP56, 1:50; Nichirei), anti-BCRP (BXP-21, 1:50; CHEMICON, Temecula, CA, USA) and anti-Ki-67 (MIB-1; 1:100; Dako Cytomation, Glostrup, Denmark). The antibody to YB-1 was anti-YBC polyclonal antibody prepared against a 15-amino acid synthetic peptide in the COOH-terminal domain.[25] This antibody was used at a working dilution of 1:100.[8] Four micrometer-thick sections were stained using a streptavidin-biotin-peroxidase method (HISTOFINE SAB-PO kit, Nichirei). For staining in the cases of all the antibodies, sections were pretreated with microwave irradiation for the purpose of antigen retrieval.

MDR human osteosarcoma cell line MNNG/HOS/DXR 1000,[26] served as a control for JSB-1, while tissue from the adrenal gland served as a control for MRPr1. Tissue from a normal liver and colon was used as a control for MRP2/cMOAT and MRP3, respectively. Moreover, tissue from normal kidney,[27] and placenta,[5] served as a control for MVP and BCRP, respectively. For each procedure, a negative control was also obtained by staining the samples with secondary antibody only.

ABC transporter proteins or LRP-positive tumor samples were graded from 0 to 3 according to the distribution of positivity and the degree of immunostaining of the plasma membrane or cytoplasmic Golgi region,[28,29] as follows: Score 0, no immunoreactive tumor cells were detected; Score 1, less than 10% of the tumor cells are positive, with weak immunostaining; Score 2, more than 10% of the tumor cells are positive, with weak immunostatining; Score 3, more than 10% of the tumor cells are positive, with strong immunoreactivity. The highest degree of positivity found in any area of the section was recorded,[28,30] and a score of 2 or 3 was judged as high expression. The immunoreactivity in each case was judged independently by three pathologists (YO, HY, ST). The MIB-1-labeling index (LI) was estimated by counting the number of positive cells per 1000 tumor cells.

Real-time quantitative RT-PCR. Total RNA was extracted using Trizol Reagent (Invitrogen Corp., Carlsbad, CA, USA) and reverse transcription was performed with Superscript II reverse transcriptase (Invtrogen Corp.) according to the manufacturer's instructions. Real-time quantitative PCR (TaqMan PCR) using an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) was performed according to the manufacturer's protocol. The sequences of oligonucleotide primer pairs and TaqMan probes for, *MRP1*, *MRP2* and *MRP3* are as previously described.[30]

Real-time quantitative PCR for *MDR1*, *MVP* and *BCRP* was performed using predeveloped TaqMan assay reagents of human *MDR1 (ABCB1)* (spanning exon 6/exon 7; ID: Hs00184500-m1), *MVP* (spanning exon 2/exon 3; ID: Hs00233856-m1), and *BCRP (ABCG2)* (spanning exon 5/exon 6; ID: Hs00184979-m1). Primers and probes for *GAPDH* were purchased from Perkin-Elmer Applied Biosystems (TaqMan GAPDH control reagent kit). All the reactions for standard samples and samples of patients were performed in triplicate. The data were averaged from the values obtained in each reaction. The mRNA levels of each of the genes were standardized by *GAPDH* and estimated as previously described.[30]

Statistical analysis. The difference in mRNA expression between tumor tissue and skeletal muscle as a control, and the correlation between real-time quantitative RT-PCR and immunohistochemistry were evaluated by the Mann–Whitney U and Kruskal–Wallis test. Association between two variables was evaluated by a two-sided chi-square test. The difference in MIB-1-LI between the two groups was estimated by unpooled two-sample *t*-test. The outcome of differences in various factors was compared by the log-rank test. Multivariate survival analysis was performed with a Cox proportional hazards regression model. A P of less than 0.05 was considered statistically significant.

Results

Patient characteristics. The clinical and pathological data for the patients with RMS are summarized in Table 1. There were

Table 1. Clinical characteristics of 35 ERMS and 28 ARMS patients

Characteristics	ERMS	ARMS
Age		
<16 years (n = 41)	27	14
≥16 years (n = 22)	8	14
Gender		
Male (n = 31)	16	15
Female (n = 32)	19	13
Anatomic site of the primary tumor		
Favorable site: (n = 16)	11	5
Orbit	0	1
Head and neck (excluding parameningeal)	3	4
GU-Nonbladder/Nonprostate	8	0
Unfavorable site: (n = 47)	25	22
Bladder/Prostate	7	0
Extremity	7	10
Head and neck, paramenigeal	2	6
Other (trunk, retroperitoneum, etc.)	9	6
Tumor size		
<5 cm (n = 21)	11	10
≥5 cm (n = 37)	23	14
Unknown (n = 5)	1	4
IRS Stage		
Stage 1 (n = 14)	9	5
Stage 2 (n = 5)	4	1
Stage 3 (n = 37)	21	16
Stage 4 (n = 2)	0	2
Unknown (n = 5)	1	4

GU, genitourinary system; IRS, Intergroup Rhabdomyosarcoma Study. ERMS, embryonal rhabdomyosarcoma; ARMS, alveolar rhabdomyosarcoma.

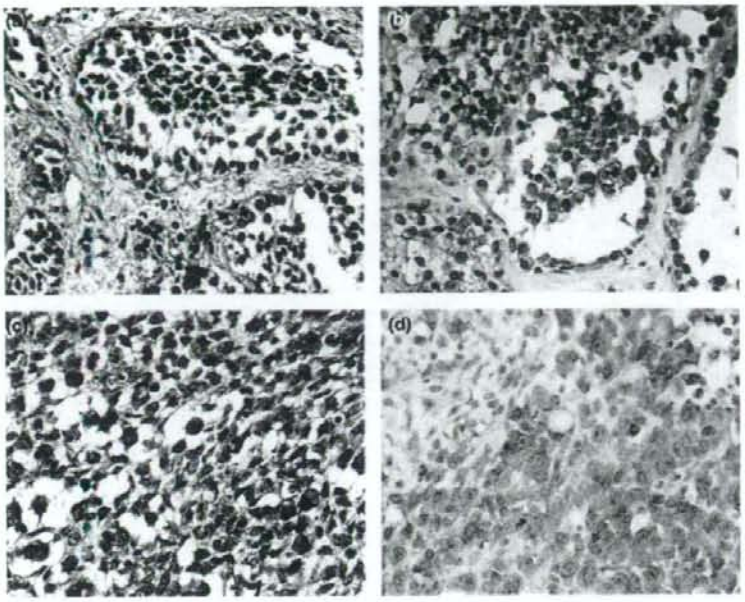

Fig. 1. Immunohistochemical expression of ATP-binding cassette transporters in rhabdomyosarcoma. Alveolar rhabdomyosarcoma arising in the nasal cavity of a 28-year-old man (a) shows strong membranous immunoreaction for P-glycoprotein (b; Score 3). Embryonal rhabdomyosarcoma in the retroperitoneum of a 4-year-old girl (c) shows both membranous and cytoplasmic localization of breast-cancer resistant protein (d; Score 3).

31 male and 32 female patients, ranging in age from 15 days to 39 years (mean, 11.0 years). Forty-one patients were children, while 22 patients were adults. Histologically, 35 tumors were classified as embryonal type, including three botryoid type and four spindle cell type. Twenty-eight tumors were categorized as alveolar type.

Immunohistochemical expression of ABC transporters, LRP and the expression of their mRNAs. Immunohistochemically, high expression of P-gp was observed in 32 out of 63 cases (51%, Fig. 1a,b). High expression of MRP1 protein was identified in 33 out of 63 cases (52%), whereas high MRP2/cMOAT expression was found in 22 cases (35%). Twenty-five tumors showed high MRP3 protein expression (40%). High expression of MVP and BCRP (Fig. 1c,d) protein was recognized in 30 (48%) and 22 (35%) cases, respectively. Concerning histological subtype, ARMS showed significantly more frequent P-gp expression (Fig. 1a,b), compared with ERMS (Table 2, $P = 0.0332$), whereas the expression of BCRP was significantly higher in ERMS (Fig. 1c,d) in comparison with that in ARMS (Table 2, $P = 0.0184$).

Tumor tissue expressed *MDR1* mRNA at a significantly higher level (57.12 arbitrary units [A.U.], mean) than the control skeletal muscle tissue (0.31 A.U., mean) ($P = 0.0429$). The levels of *MRP1* (mean, 33.47 A.U.), *MRP3* (mean, 6210.6 A.U.) and *LRP* (mean, 980.13 A.U.) mRNA expression in tumor tissue were also significantly higher than those in the control tissue (*MRP1*: mean, 0.85 A.U., $P = 0.0234$, *MRP3*: mean, 0.54 A.U., $P = 0.0004$, *LRP*: mean, 0.09 A.U., $P = 0.0003$). Concerning *MRP2* and *BCRP* mRNA expression, no statistically significant difference was recognized between tumor tissue and the control tissue. When the results from immunohistochemistry and real-time quantitative RT-PCR techniques were compared, a statistical association was found between immunoreactivities and mRNA expression levels for all ABC transporters and *LRP* (Table 3 and Fig. 2).

YB-1 protein expression. All 63 cases of RMS showed positive immunoreaction for anti-YBC in the cytoplasm with uniform intensity (Fig. 3a). In 24 of the 63 cases (38%), YB-1 expression was also recognized in the nucleus (Fig. 3b). YB-1 nuclear

Table 2. Comparison of immunohistochemical results between embryonal and alveolar rhabdomyosarcoma

Protein		ERMS ($n = 35$)	ARMS ($n = 28$)	P-value
YB-1	Nuclear	12	12	$P = 0.3313$
	Cytoplasm	23	16	
P-gp	Score 0	10	3	$P = 0.0332$*
	1	13	5	
	2	6	9	
	3	6	11	
MRP1	Score 0	14	4	$P = 0.0713$
	1	4	8	
	2	9	6	
	3	8	10	
MRP2	Score 0	8	9	$P = 0.1761$
	1	16	8	
	2	4	8	
	3	7	3	
MRP3	Score 0	10	7	$P = 0.3645$
	1	13	8	
	2	9	6	
	3	3	7	
MVP	Score 0	11	6	$P = 0.4957$
	1	10	6	
	2	8	7	
	3	6	9	
BCRP	Score 0	5	14	$P = 0.0184$*
	1	14	8	
	2	6	3	
	3	10	3	
MIB-1-LI		18.26 ± 13.59	12.23 ± 7.71	$P = 0.0407$*

*Statistically significant.
ARMS, alveolar rhabdomyosarcoma; BCRP, breast-cancer resistant protein; ERMS, embryonal rhabdomyosarcoma; MIB-1-LI, MIB-1-labeling index; MRP, multidrug resistance-associated protein; MVP, major vault protein; P-gp, P-glycoprotein; YB-1, Y-box-binding protein-1.

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Table 3. Correlation between protein and mRNA expression

Protein		mRNA (A.U.) mean	SD	MW-test**	KW-test***
P-gp	Score 0 or 1 ($n = 9$)	0.579	0.909	$P = 0.0003$*	$P = 0.0037$*
	Score 2 or 3 ($n = 9$)	113.656	156.308		
MRP1	Score 0 or 1 ($n = 5$)	1.422	1.402	$P = 0.0137$*	$P = 0.0077$*
	Score 2 or 3 ($n = 13$)	45.79	52.754		
MRP2	Score 0 or 1 ($n = 9$)	0.67	1.262	$P = 0.0152$*	$P = 0.0222$*
	Score 2 or 3 ($n = 9$)	8.532	14.628		
MRP3	Score 0 or 1 ($n = 6$)	208.669	301.488	$P = 0.0159$*	$P = 0.0057$*
	Score 2 or 3 ($n = 11$)	9484.374	21 576.062		
MVP	Score 0 or 1 ($n = 10$)	112.14	239.27	$P = 0.033$*	$P = 0.0075$*
	Score 2 or 3 ($n = 8$)	2065.115	3995.216		
BCRP	Score 0 or 1 ($n = 7$)	0.113	0.157	$P = 0.0063$*	$P = 0.0142$*
	Score 2 or 3 ($n = 11$)	32.538	50.236		

*Statistically significant, **Mann–Whitney (MW) *U*-test (comparison of two groups/Score 0 or 1 *versus* Score 2 or 3); ***Kruskal–Wallis (KW) test in four groups (Score 0, 1, 2, 3). A.U., arbitrary units; BCRP, breast-cancer resistant protein; MRP, multidrug resistance-associated protein; MVP, major vault protein; P-gp, P-glycoprotein; SD, standard deviation.

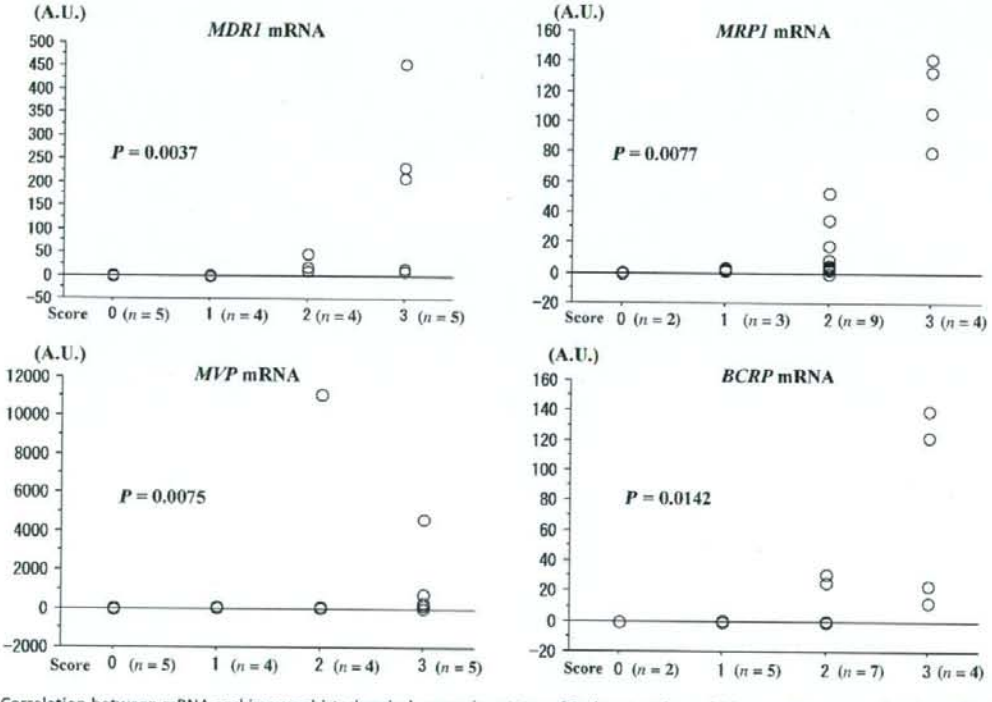

Fig. 2. Correlation between mRNA and immunohistochemical expression status of P-glycoprotein, multidrug resistance-associated protein (MRP)1, major vault protein (MVP) and breast-cancer resistant protein (BCRP). The immunohistochemical status was significantly correlated with the concordant mRNA expression ($P < 0.05$, Kruskal–Wallis test). A.U., arbitrary units.

expression was observed in 12 of 35 ERMS (34%), whereas it was recognized in 12 of 28 ARMS (43%). In ERMS, significant correlation was also recognized between YB-1 nuclear expression and P-gp ($P = 0.0359$) or MVP ($P = 0.0044$) (Table 4). On the other hand, in ARMS no such relationship was observed.

MIB-1-li. In ERMS, the cases with YB-1 nuclear expression also showed a significantly higher MIB-1-LI (mean, 25.299), compared with the cases with cytoplasmic YB-1 expression (mean, 14.584) (Table 4, $P = 0.0244$). However, no such difference was observed in ARMS. Moreover, the MIB-1-LI of the cases with high BCRP expression was significantly higher (mean, 19.88) than that of the cases without high BCRP expression (mean, 13.27) ($P = 0.0311$).

Survival analysis. The results of survival analysis are summarized in Table 5. Adult patients showed significantly poor prognosis, compared with children ($P = 0.0125$). The cases with an unfavorable site ($P = 0.0739$), a high IRS stage (stage 3 or 4) ($P = 0.0843$) showed poor survival by univariate analysis, however, these results showed no statistical significance. On the

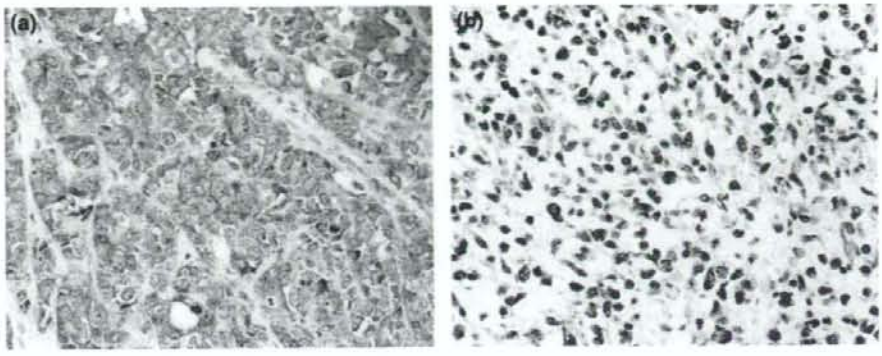

Fig. 3. (a) Alveolar rhabdomyosarcoma arising in the vulva of a 21-year-old woman. Y-box-binding protein-1 (YB-1) expression is observed only in the cytoplasm. This tumor showed negative immunoreactivity for P-glycoprotein (P-gp) (Score 0). (b) Embryonal rhabdomyosarcoma arising in the testis of a 14-year-old boy. Diffuse and strong nuclear expression of YB-1 protein is evident in the tumor cells. This tumor showed high expression of P-gp (Score 3).

other hand, multivariate analysis including clinicopathological and immunohistochemical parameters revealed age at diagnosis (≥16 years old: $P = 0.0072$), MRP1 ($P = 0.0244$) and MRP2 ($P = 0.0326$) expressions were an independent and significant factors for poor prognosis.

Table 4. Correlation between YB-1 nuclear expression and P-glycoprotein or MVP expression, and MIB-1-LI

		Embryonal RMS		
	YB-1	Nuclear	Cytoplasm	
P-gp	Score 0	2	8	$P = 0.0359$*
	1	2	11	
	2	4	2	
	3	4	2	
MVP	Score 0	0	11	$P = 0.0044$*
	1	3	7	
	2	4	4	
	3	5	1	
		Alveolar RMS		
	YB-1	Nuclear	Cytoplasm	
P-gp	Score 0	1	2	$P = 0.9827$
	1	2	3	
	2	4	5	
	3	5	6	
MVP	Score 0	3	3	$P = 0.494$
	1	1	5	
	2	3	4	
	3	5	4	
		Embryonal RMS		
	MIB-1-LI	Mean	SD	
YB-1	N ($n = 12$)	25.299	15.298	$P = 0.0244$*
	C ($n = 23$)	14.584	11.274	
		Alveolar RMS		
	MIB-1-LI	Mean	SD	
YB-1	N ($n = 12$)	14.501	6.278	$P = 0.1822$
	C ($n = 16$)	10.528	8.424	

*Statistically significant. C, cytoplasmic; MIB-1-LI, MIB-1-labeling index; MVP, major vault protein; P-gp, P-glycoprotein; N, nuclear; RMS, rhabdomyosarcoma; SD, standard deviation; YB-1, Y-box-binding protein-1.

Discussion

There have been some investigations concerning the expression of MDR1/P-gp or MRP1 in a large series of RMS patients.[23,31–33] Gallego *et al.*[32] demonstrated frequent *MDR1* and *MRP1* mRNA expression in ARMS compared with ERMS, whereas other studies,[23,31] have failed to reveal any differences in P-gp or MRP1 protein expression between ARMS and ERMS. In the current study, ARMS showed significantly frequent P-gp expression in comparison with ERMS. Frequent chemoresistant phenotype in ARMS may be due to frequent P-gp expression. Moreover, in the current study, MRP1 expression was one of the adverse prognostic factors, using multivariate analysis.

As for MVP expression in RMSs, Komdeuer *et al.*[23] reported either no expression or only limited expression of MVP in ARMS compared with ERMS. Moreover, their group also demonstrated that MVP expression was most prominent in the more differentiated tumor cells in untreated tumors and that its expression increased significantly following chemotherapy.[33] Accordingly, they concluded that MVP plays some part in therapy-induced differentiation. In our series, MVP expression was recognized in both undifferentiated and differentiated tumor cells, and no difference was observed between ERMS and ARMS with regard to MVP expression.

BCRP (ABCG2) also belongs to the ABC transporter family and its sequence is similar to one-half of the duplicated P-gp or MRP1 molecule.[5] BCRP affects a narrow range of anticancer agents compared to the MDR1 (ABCB1) and MRP (ABCC) transporters, including anthracyclines, mitoxantrone and topoisomerase I inhibitors.[34] Although a close correlation between the overexpression of BCRP and poor prognosis has been reported in adult acute myeloid leukemia,[35] there have been no investigations of BCRP expression in malignant pediatric solid tumors, including RMS. In this study, BCRP expression was significantly more frequently observed in ERMS compared with ARMS. Moreover, the cases with high BCRP expression showed higher proliferating activities as measured by the MIB-1-LI.

Previous studies have demonstrated a close relationship between the nuclear expression of YB-1 and MDR1/P-gp expression in several kinds of human malignancies.[7–9] We previously demonstrated that YB-1 nuclear expression also had a significant correlation with high proliferative activities as determined by the MIB-1-LI in human osteosarcoma.[8] Stein *et al.*[10] demonstrated an increased expression of endogenous MVP protein by transduction of YB-1 cDNA *in vivo*, and a strong correlation between MVP and YB-1 expression in human colon

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Table 5. Overall survival in 61 cases of rhabdomyosarcoma

Variable		P-value in survival analysis	
		Univariate	Multivariate
Clinicopathologic			
Age	<16 years (n = 39) ≥16 years (n = 22)	0.0065*	0.0072*
Sex	Male (n = 30) Female (n = 31)	0.8254	0.0979
Site	Favorable (n = 15) Unfavoable (n = 46)	0.0755	0.4612
Histology	Embryonal (n = 33) Alveolar (n = 28)	0.2896	0.6479
Size	<5 cm (n = 20) ≥5 cm (n = 36)	0.214	0.0662
Stage	Low (1,2) (n = 18) High (3,4) (n = 38)	0.0914	0.3853
Immunohistochemical			
	YB-1 nuclear expression (–) (n = 37) (+) (n = 24)	0.4431	0.0538
	P-gp (Score 0 or 1) (n = 30) (Score 2 or 3) (n = 31)	0.1339	0.0948
	MRP1 (Score 0 or 1) (n = 30) (Score 2 or 3) (n = 31)	0.1397	0.0244*
	MRP2 (Score 0 or 1) (n = 39) (Score 2 or 3) (n = 22)	0.775	0.0326*
	MRP3 (Score 0 or 1) (n = 37) (Score 2 or 3) (n = 24)	0.707	0.298
	MVP (Score 0 or 1) (n = 32) (Score 2 or 3) (n = 29)	0.3501	0.1958
	BCRP (Score 0 or 1) (n = 41) (Score 2 or 3) (n = 20)	0.4945	0.4907
	MIB-1 LI (<15.7, mean) (n = 36) (≥15.7, mean) (n = 25)	0.1535	0.1169

*Statistically significant. BCRP, breast-cancer resistant protein; MIB-1-LI, MIB-1-labeling index; MRP, multidrug resistance-associated protein; MVP, major vault protein; P-gp, P-glycoprotein; YB-1, Y-box-binding protein-1.

cancer specimens. Moreover, we recently reported a close relationship between YB-1 nuclear expression and MVP expression in human ovarian cancer.[11] In the current study, significant relationships between YB-1 nuclear expression and P-gp/MVP expression, or a high MIB-1-LI were observed in only ERMS, with no such correlation being recognized in ARMS. On the other hand, P-gp expression was more frequently observed in ARMS compared with ERMS. Therefore, in ARMS, mechanism behind the up-regulation of P-gp may not be due to the YB-1 pathway and it may be quite different from that in ERMS. These different expression profiles of MDR-related proteins and YB-1 nuclear expression support the concept that ARMS is a distinct tumor which is molecular genetically different from ERMS,[16] except with regard to skeletal muscle differentiation which is manifest in both tumors.

The nuclear expression of YB-1 is reported to be associated with poor prognosis in several kinds of malignant solid tumors.[1,2,11] There have been no investigations concerning YB-1 nuclear expression in pediatric malignant solid tumors. In this study, YB-1 nuclear expression did not show any prognostic significance. Moreover, we could not find correlation between histological type, site and size with survival. These factors have been reported as predictive prognostic factors in RMS.[13] These discrepancies may be due to the fact the patients in this series were in heterogeneously treated groups. To elucidate the correlation between YB-1 nuclear expression and prognosis in RMS, further large studies in uniformly treated groups are needed.

In conclusion, ARMS and ERMS showed different expression profiles of MDR-related molecules and this result supports the theory that both tumors are molecular genetically distinct. YB-1 could be novel candidate for a therapeutic target, especially in cases of ERMS, because of the close correlation between YB-1 nuclear expression and P-gp in this tumor.

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Disclosure/conflict of interest

The authors declare no disclosures or conflicts of interest.

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Inhibition of bone and muscle metastases of lung cancer cells by a decrease in the number of monocytes/macrophages

Koji Hiraoka,[1,2,7] Michihisa Zenmyo,[1,2] Kousuke Watari,[3] Haruo Iguchi,[4] Abbas Fotovati,[2] Yusuke N. Kimura,[2] Fumihito Hosoi,[2] Takanori Shoda,[1,2] Kensei Nagata,[1,2] Hiroyuki Osada,[5] Mayumi Ono[3,6] and Michihiko Kuwano[2,6]

[1]Department of Orthopedic Surgery and [2]Research Center of Innovative Cancer Therapy, Kurume University School of Medicine, Kurume 830-0011; [3]Department of Pharmaceutical Oncology, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582; [4]Clinical Research Institute, Shikoku Cancer Center, Matsuyama 791-0280; [5]Antibiotics Laboratory, Discovery Research institute, RIKEN, Saitama 351-0198; [6]Innovative Center for Medical Redox Navigation, Kyushu University, Fukuoka 812-8582, Japan

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Attention has recently focused on the critical role of inflammatory responses in the tumor stroma that provide favorable conditions for cancer-cell growth and invasion/metastasis. In particular, macrophages recruited into the tumor stroma and activated, known as tumor-associated macrophages, are suggested to promote tumorigenesis. In this study, we examined the effect of a decrease in the number of monocytes/macrophages in peripheral blood and the tumor stroma on the development of bone and muscle metastases by lung cancer cells. Treatment with clodronate encapsulated by liposomes (Cl_2MDP-LIP) has been developed for the depletion of monocytes/macrophages in an animal model. Subcutaneous administration of Cl_2MDP-LIP markedly reduced the number of monocytes in peripheral blood, resulting in efficient suppression of both bone metastasis and muscle metastasis when lung cancer HARA-B cells were injected into the left cardiac ventricle of mice. Treatment with Cl_2MDP-LIP significantly reduced the number of macrophages in tumors and the number of osteoclasts in bone marrow, as well as peripheral monocytes in mice harboring lung cancer cells. In contrast, treatment with an osteoclast-targeting antibiotic, reveromycin A, inhibited bone metastasis by lung cancer cells, but not muscle metastasis. The survival of human macrophages in culture was found to be specifically blocked by Cl_2MDP-LIP, but not by reveromycin A. Cl_2MDP-LIP thus exerted antimetastatic effects in both bone and muscle whereas reveromycin A did so only in bone. Liposome-encapsulated bisphosphonate may modulate metastasis through decreasing the number of monocytes/macrophages in both peripheral blood and the tumor stroma, suggesting that tumor-associated macrophages might be suitable targets for antimetastatic therapy. (*Cancer Sci* 2008; 99: 1595–1602)

Metastases of several malignant cancers including those of the breast, lung, prostate, and kidney have high affinity for bone. Bone metastasis is often accompanied by serious complications such as pathological fractures, bone pain, spinal cord compression, and hypercalcemia. Organ metastasis, including that affecting bone, is a multistep process mediated through mutual interaction between cancer cells and the host microenvironment. In bone metastasis, cancer cells reach the bone via hematogenous spread, followed by osteoclastic bone resorption, and finally proliferate in the bone matrix.[1,2] Moreover, osteoclast-stimulating cytokines such as PTHrP have been shown to promote bone metastasis.[3]

Inflammatory responses in the tumor stroma play an important role by providing favorable conditions for cancer cell growth, invasion/metastasis, and angiogenesis as well as malignant progression.[4–6] In particular, monocytes/macrophages are recruited into the tumor stroma, and activated macrophages known as TAMs produce potent angiogenic factors, as well as inflammatory cytokines, growth factors, and proteases, resulting in a promotion of angiogenesis and invasion/metastasis.[7–9] Infiltrating TAMs are often closely associated with poor prognosis and tumor angiogenesis in patients with various tumor types.[9–11] A preparation of Cl_2MDP-LIP has been reported to markedly inhibit angiogenesis in corneas in response to inflammatory cytokines through depletion of macrophages.[12] A recent study has demonstrated that administration of clodronate-liposomes depleted TAMs in mouse models resulting in significant inhibition of tumor growth and tumor angiogenesis, whereas free clodronate alone did not.[13] Clodronate-liposomes were also found to inhibit both tumor growth and tumor angiogenesis by lung cancer cells in a xenograft model when stimulated by inflammatory stimuli.[14] Angiogenesis in a tumor microenvironment in bone marrow also played a critical role in the induction of an angiogenic response and invasion/metastasis by cancer cells.[15] Furthermore, monocyte/macrophage precursor cells entered the osteoclastic lineage and expressed the osteoclastic marker TRAP under the influence of the RANK/RNAKL signaling pathway.[16] Tumor burden at bone metastatic sites was markedly decreased in preclinical models on treatment with inhibitors of the RANK/RANKL pathway and neutralizing antibodies against PTHrP as well as bisphosphonate, suggesting a central role for osteoclasts in bone metastasis.[17–19] Together, one can expect a decrease in the number of the monocyte/macrophage-lineage by clodronate-liposomes to attenuate the bone metastasis and growth by cancer cells.

In the present study, using an animal model of bone metastasis with the human lung cancer cell line HARA-B, we investigated whether the administration of clodronate-liposomes was able to modulate bone metastasis by lung cancer cells.[3,20] On the basis of our results, we discuss whether liposome-encapsulated bisphosphonate may be useful for treating not only bone metastasis from lung cancer, but also metastasis in other tissues/organs.

Materials and Methods

Cell culture. HARA-B cells were established from bone metastasis of human lung cancer in nude mice and cultured in RPMI-1640 supplemented with 10% FBS and 10-U/mL

[7]To whom correspondence should be addressed.
E-mail: khiraoka@med.kurume-u.ac.jp

Abbreviations: Ab, antibody; BSA, bovine serum albumin; Cl_2MDP-LIP, clodronate encapsulated by liposomes; DAPI, 4',6-diamidino-2-phenylindole; FITC, fluorescein-isothiocyanate; HRP, horseradish peroxidase; OCT, optimal cutting temperature; PBS, phosphate-buffered saline; PE, phycoerythrin; PTHrP, parathyroid hormone-related protein; RT, room temperature; TAMs, tumor-associated macrophages; TRAP, tartrate-resistant acid phosphatase.

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penicillin-streptomycin.[3,20] The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. Human macrophage-like cell line U937 was purchased from the American Type Culture collection (Manassas, VA, USA) and cultured in RPMI supplemented with 10% FBS.

Reagents. FITC-conjugated anti-F4/80 mAb and PE-conjugated anti-CD11b mAb were obtained from CALTAG Laboratories (Burlingame, CA, USA). Rat antimouse F4/80 Ab (MAC497R) was obtained from Serotec (Raleigh, NC, USA). Rat antimouse Gr-1 was purchased from R&D Systems (Minneapolis, MN, USA). Phosphatidylcholine, cholesterol, and clodronate (dichloromethylene diphosphate; Cl_2MDP) were from Sigma-Aldrich (St. Louis, MO, USA). Reveromycin A was a gift from Riken (Saitama, Japan).

Preparation of Cl2MDP-LIP. Cl_2MDP-LIP was prepared as described previously.[12,21] A total of 11 mg of cholesterol and 75 mg of phosphatidylcholine were combined with 10 mL of 0.7-M Cl_2MDP solution and sonicated gently. The resulting liposomes were washed three times to eliminate any free drug. Empty liposomes were prepared as a control under the same conditions using PBS instead of Cl_2MDP.

Animals. Female 5-week-old BALB/C nude mice were obtained from Clea Japan (Tokyo, Japan) and maintained in a specific pathogen-free environment throughout the experiment.

Flow cytometry. Blood samples were obtained from the left cardiac ventricle in mice under anesthesia at day 0, 1, and 2 after stimulation with Cl_2MDP-LIP. A total of 50 mL of each sample was stained for 15 min in a dark doom with FITC-anti-F4/80 mAb (1:50) to label macrophages, and with PE-anti-CD11b mAb (1:50) to label macrophages and neutrophils. Positive cells were measured using a FACScan (Becton Dickinson, USA).[12]

Bone and muscle metastasis by cancer cells, and antimetastatic therapeutic protocol. HARA-B cells (2×10^5/100 μL) were injected into the left cardiac ventricle of mice on day 0 under anesthesia with pentobarbital (0.05 mg/g body weight; Dainippon Pharmaceutical, Osaka, Japan).[3] To assess the inhibitory effect of Cl_2MDP-LIP on the formation of bone and muscle metastasis, Cl_2MDP-LIP at 200 μL or 400 μL was subcutaneously (s.c.) administered into the base of the tail once every 3 days for 6 weeks after the inoculation of HARA-B cells. A subcutaneous administration of reveromycin A (10 mg/kg) was also performed every day for 6 weeks after the inoculation of HARA-B cells. Bone metastases were determined on X-ray photographs at 4 or 6 weeks. Osteolytic bone metastasis on X-ray photographs was evaluated independently. Mice were sacrificed under anesthesia with pentobarbital (0.5 mg/g body weight) at 5 or 6 weeks after inoculation. The extremities and spine were harvested and fixed in 10% formalin. The bone specimens were decalcified in 10% EDTA solution for 1 week and then embedded in paraffin. Tumor metastases were histologically evaluated by the number of colonies and the tumor area in bone and muscle after hematoxylin–eosin staining.[22]

Immunohistochemical and immunofluorescence analysis. Macrophages in bone marrow and tumors were determined using immunohistochemistry for F4/80. Slides were deparaffinized and hydrated, and then rinsed twice with PBS. After 1 h of blocking with 2% goat serum, the sections were incubated overnight with rat antimouse F4/80 (1:200) at 4°C in 1% BSA in PBS. They were then rinsed three times with PBS and treated with HRP-conjugated goat antirat IgG (DakoCytomation, CA, USA) and stained using the DakoCytomation LSAB2 SYSTEM HRP kit, according to the instructions. The sections were counterstained with diluted hematoxylin according to the manufacturer's directions.

For the detection of osteoclasts, TRAP staining was done using a Sigma Diagnostics Acid Phosphatase kit. The number of TRAP-positive cells in bone marrow was counted by microscopy in five random fields in each of three sections at ×200 magnification.

To determine the macrophages and neutrophils in HARA-B tumors, immunofluorescence staining was performed. The tumor samples from bone or muscle were excised and immersed in OCT compound, and immediately frozen in liquid nitrogen. Frozen sections 5-μm thick were prepared. The sections were rinsed with PBS and briefly fixed in 4% paraformaldehyde/PBS for 20 min at RT, followed by two further rinses in PBS. After 1 h of blocking with 2% goat serum, the sections were incubated overnight with rat antimouse $F_{4/80}$ (1:200) or rat antimouse Gr-1 (1:200) at 4°C in 1% BSA in PBS. They were then rinsed three times with PBS and incubated with goat antirat IgG; 1-mg/mL Alexa Fluor 488 for F4/80 (Molecular Probes, Eugene, OR, USA) in 1% BSA in PBS for 60 min at RT. Nuclear staining was carried out using DAPI (1:1000; Dojindo, Japan). Coverslips were mounted on sections using gel mount and viewed using an Olympus BX51 florescence microscope (Olympus, Tokyo, Japan) fitted with an Olympus DP-70 digital camera (Olympus). For quantification, the number of stained cells was counted in five random fields in each of three tumors at ×200 magnification.

Cell survival assay. Cell survival assay was carried out using a Cell Counting Kit (Wako Pure Chemical Industries, Osaka, Japan). In brief, HARA-B cells and human monocytes were plated in triplicate in 96-well plates at a density of 5000 cells/well in basal medium. Following overnight culture, PBS-LIP, Cl_2MDP-LIP, or reveromycin A was added to each concentration, and the cells were incubated for 48 h. After 48 h, WST-1 was added and the cells were incubated for a further 1 h. The plates were read at a wavelength of 450 nm using a microplate reader (Model 3550; Bio-Rad, Richmond, CA, USA). Results are presented as the mean ± SD.

Statistical analysis. The significance of tumor incidence was determined by χ^2-test. The significance of differences in the number of metastases was determined by the Mann–Whitney *U*-test. The significance of differences in the numbers of TRAP-positive cells and tumor area was estimated using the unpaired Student's *t*-test. *P*-values of <0.05 were considered statistically significant.

Results

Decrease in the number of monocytes/macrophages by Cl_2MDP-LIP *in vitro* and *in vivo*. Blood samples were harvested from the left cardiac ventricles of mice under anesthesia as a control. Subsequently, Cl_2MDP-LIP at 200 μL and 400 μL was s.c. administered into the base of the tail, and a 100-μL blood sample was obtained after 24 and 48 h. Each sample was stained with FITC-anti-F4/80 mAb and PE-anti-CD11b mAb to label macrophages, and double-positive cells were measured using a FACScan. The rate of double-positive staining was about 5–7% before stimulation with Cl_2MDP-LIP. The number of double-positive cells in peripheral blood was decreased significantly at 24 h after stimulation with 400 μL of Cl_2MDP-LIP but had recovered slightly after 48 h. Although stimulation with 200 μL of Cl_2MDP-LIP also suppressed the percentage of monocytes at 24 h, the effect was less marked than that of 400 μL of Cl_2MDP-LIP (Fig. 1a,b). PBS-LIP and reveromycin A did not decrease the number of monocytes in peripheral blood (Fig. 1c). We also compared the effects of Cl_2MDP-LIP and reveromycin A on the survival of lung cancer HARA-B cells and macrophage U937 cells in culture. Survival of cancer cells was specifically inhibited by reveromycin A at both 5 and 10 μg/mL, but Cl_2MDP-LIP had no effect up to 200 μM (Table 1). By contrast, survival of macrophages was blocked only by 20–200 μM Cl_2MDP-LIP, but not by reveromycin A at up to 10 μg/mL (Table 1). Thus, Cl_2MDP-LIP had a more specific effect on

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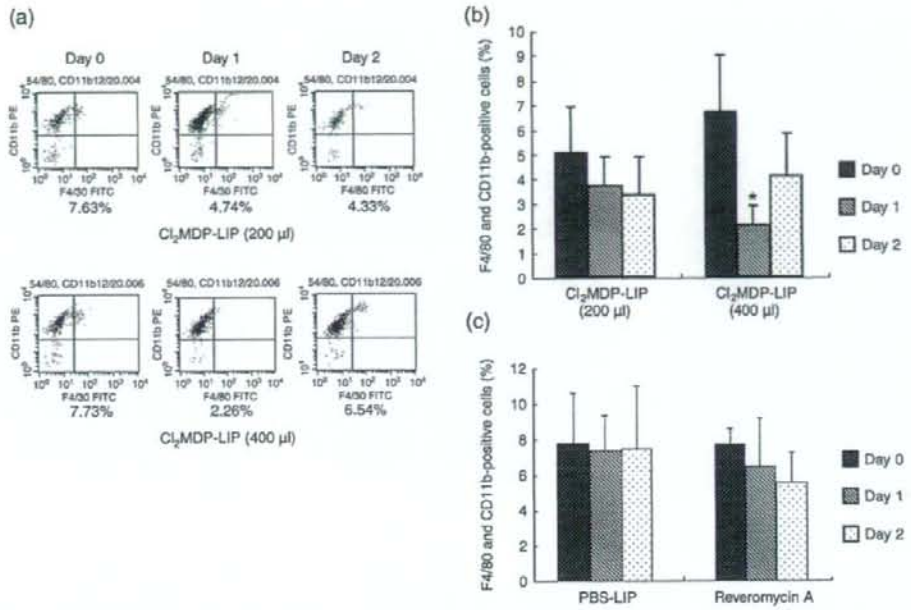

Fig. 1. The decrease in the number of monocytes in peripheral blood by treatment with clodronate encapsulated by liposomes (Cl_2MDP-LIP). (a) FACS analysis of macrophages on days 0, 1, and 2 in peripheral blood of nude mice untreated or treated with Cl_2MDP-LIP. Each blood sample (100 μL) was harvested from the left cardiac ventricle under anesthesia before stimulation. Subsequently, blood samples were taken on days 1 and 2 after the subcutaneous administration of Cl_2MDP-LIP at 200 μL or 400 μL/mouse. Cells were stained with fluorescein-isothiocyanate (FITC)–anti-F4/80 monoclonal antibody (mAb) (1:50) and phycoerythrin (PE)–anti-CD11b mAb (1:50). F4/80- and CD11b-positive cells were measured by FACScan. Upper lane, Cl_2MDP-LIP 200 μL; lower lane, Cl_2MDP-LIP 400 μL. Quantification of the number of monocytes in peripheral blood of nude mice treated with (b) Cl_2MDP-LIP at 200 μL or 400 μL, and (c) PBS-LIP (400 μL) or reveromycin A (10 mg/kg). Each value represents the mean number of monocytes/macrophages ± SD ($n = 4$). $^*P < 0.05$.

Table 1. Effect of clodronate, Cl_2MDP-LIP, and reveromycin A on the survival of macrophages and lung cancer cells

Drug	Dose	Lung cancer cells†	Macrophages†
PBS-LIP	0	100.0 ± 4.0	100.0 ± 5.9
Clodronate	2	93.3 ± 5.6	98.3 ± 6.6
	20	93.8 ± 7.1	99.9 ± 11.5
	200 (μM)	99.1 ± 1.8	92.7 ± 8.9
Cl_2MDP-LIP	2	100.0 ± 2.6	94.0 ± 9.1
	20	100.0 ± 0.4	74.7 ± 2.4
	200 (μM)	100.0 ± 5.7	26.4 ± 3.7
Reveromycin A	1	100.0 ± 5.6	101.2 ± 1.6
	5	33.1 ± 2.5	100.7 ± 2.4
	10 (μg/mL)	12.2 ± 0.4 (%)	105.3 ± 1.5 (%)

HARA-B cells (5×10^3/well) and macrophages (5×10^3/well) were incubated for 2 days in the absence or presence of various doses of drugs, and surviving fractions were determined.
Each value was the average of triplicate dishes, and presented as a relative percentage, the cell number in the absence of any drug being taken as 100%. †Mean ± SD.

macrophage survival than reveromycin A in both *in vitro* and *in vivo*.

Inhibition of bone and muscle metastases by Cl2MDP-LIP. Bone and muscle metastases in mice were followed using X-ray photographs at 4 or 6 weeks after inoculation of 2×10^5 cancer cells (Fig. 2a). Colonies of abundant proliferating cancer cells were observed in both bone and muscle when both regions were histologically examined at 6 weeks after cancer cell inoculation (Fig. 2b). We first examined the inhibitory effect of Cl_2MDP-LIP on bone and muscle metastases of cancer cells in comparison with PBS-LIP (control). Based on the effects of Cl_2MDP-LIP (Fig. 1), we determined the protocols shown in Fig. 3a. Cl_2MDP-LIP was administered s.c. at 200 μL and 400 μL once every 3 days for 6 weeks just after the inoculation of cancer cells (Fig. 3a). All of the mice treated with PBS-LIP showed destructive bone changes in X-ray photographs or paralysis in the hind limbs at 6 weeks. By contrast, treatment with Cl_2MDP-LIP at 200 μL and 400 μL inhibited the development of bone metastases by cancer cells (Fig. 3b). Fig. 3b also shows the therapeutic effects of reveromycin A on bone metastasis when administered s.c. at 10 mg/kg daily after cancer cell inoculation. Treatment with reveromycin A markedly inhibited bone metastasis by lung cancer cells. Quantitative analysis showed that both the incidence of bone metastasis and the number of metastatic foci were significantly decreased by treatment with Cl_2MDP-LIP at 200 μL and 400 μL, and reveromycin A (Table 2). The inhibitory effect of Cl_2MDP-LIP at 400 μL was strongest among those agents (Table 2).

We also compared the therapeutic effects of various agents on both muscle metastasis and bone metastasis by histological analysis. Treatment with Cl_2MDP-LIP at 400 μL and reveromycin A significantly decreased the number of tumor colonies in bone (Table 3). We also observed a marked decrease of tumor colony numbers in muscle by Cl_2MDP-LIP at 400 μL ($P < 0.05$) but not by reveromycin A. Furthermore, Cl_2MDP-LIP at 400 μL significantly ($P < 0.05$) inhibited tumor area in bone as compared

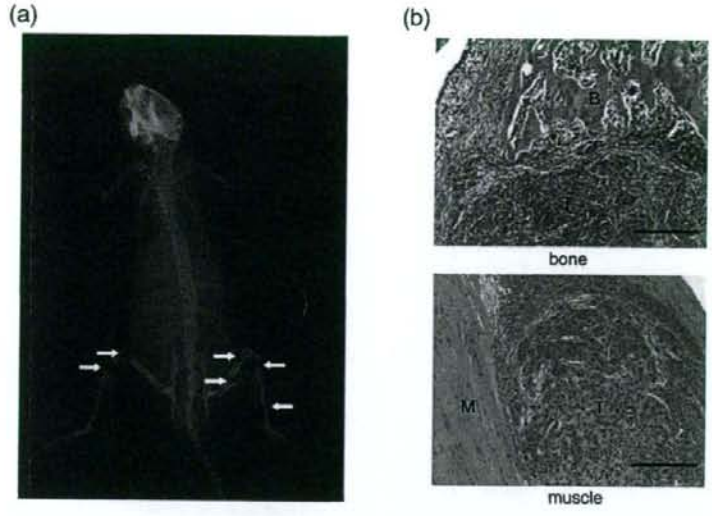

Fig. 2. Radiographic and histological analysis of untreated mice. The human lung cancer cell line HARA-B (2×10^5 cells per mouse) was injected into the left cardiac ventricle of nude mice. (a) Bone metastases were determined by radiography on the indicated days after inoculation. Arrows indicate osteolytic bone metastases. (b) The mice were sacrificed at 6 weeks, and bone and muscle metastases were examined histologically. Bar, 200 μm; T, tumor; B, bone; M, muscle.

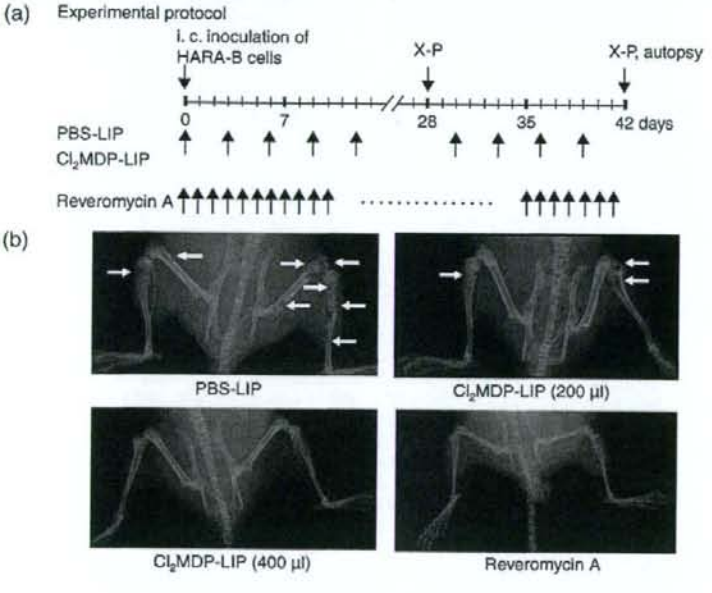

Fig. 3. Clodronate-liposomes and reveromycin A decreased the frequency of bone metastasis by lung cancer cells. (a) Experimental protocol of the treatment with clodronate encapsulated by liposomes (Cl_2MDP-LIP) and reveromycin A on bone or muscle metastases of HARA-B cells. (b) Radiographs of the hind limbs of nude mice treated with PBS-LIP (400 μL), Cl_2MDP-LIP (200 μL), Cl_2MDP-LIP (400 μL), and reveromycin A (10 mg/kg). HARA-B cells (2×10^5 per mouse) were injected into the left cardiac ventricle. At 6 weeks after the subcutaneous administration of PBS-LIP, Cl_2MDP-LIP (200 μL and 400 μL/mouse once every 3 days), and reveromycin A (every day) the extent of bone and muscle metastases was determined by radiography and autopsy. Arrows indicate osteolytic bone metastases.

Table 2. Radiological analysis of inhibitory effects of clodronate-liposomes and reveromycin A on bone metastasis

Treatment	Incidence of bone metastasis	No. of metastatic foci*
PBS-LIP	8/9	6.6 ± 4.2
Cl_2MDP-LIP (200 μl)	3/7†	0.9 ± 1.2‡
Cl_2MDP-LIP (400 μl)	1/9‡	0.1 ± 0.3‡
Reveromycin A	1/9‡	0.3 ± 1.0‡

HARA-B cells (2×10^5 per mouse) were injected into the left cardiac ventricle of nude mice on day 0. The mice were s.c. administered PBS-LIP, Cl_2MDP-LIP (200 and 400 μl/mouse once every three days), or reveromycin A (10 mg/kg daily) from day 0 to 6 weeks. Bone metastases were determined by radiographs at 4 and 6 weeks after inoculation. *Mean ±SD, †$P < 0.05$, ‡$P < 0.01$.

with the control, whereas reveromycin A or Cl_2MDP-LIP at 200 μL decreased the tumor area by only 30–50% compared with the control. In contrast, there was no significant difference in the inhibitory effect on tumor area in muscle between the untreated control and treated groups. Of the various treatments against tumor area in muscle, only Cl_2MDP-LIP at 400 μL had an inhibitory effect, although the inhibition was not statistically significant (Table 3).

Decrease in the number of both macrophages in tumor and osteoclasts in bone by Cl2MDP-LIP. We examined whether the number of macrophages was affected by treatment with Cl_2MDP-LIP. The number of macrophages in tumors was also estimated by immunofluorescence analysis with a rat antimouse F4/80 antibody. The immunofluorescence analysis of mice

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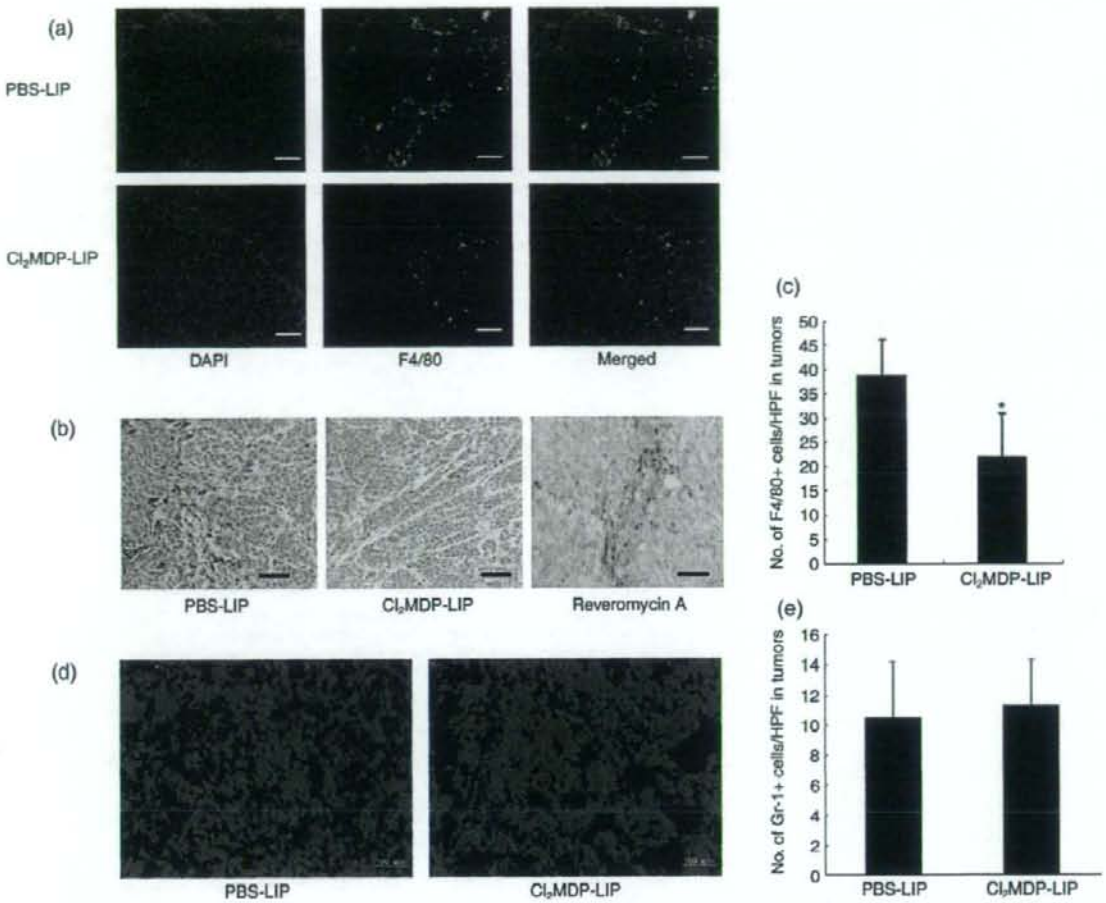

Fig. 4. Reduced infiltration of macrophages in tumors by clodronate encapsulated by liposomes (Cl_2MDP-LIP). (a) Immunofluorescence analysis of F4/80-positive cells in HARA-B tumors. The tumors derived from mice treated with PBS-LIP, Cl_2MDP-LIP (400 μL), and reveromycin A (10 mg/kg) were excised at 6 weeks after inoculation as described in the experiment protocol. The samples were stained with rat anti-F4/80 antibody (1:200), and ant-rat Alexa-Fluor-488 (1:1000) was used as secondary antibody to detect the F4/80-positive macrophages. Nuclear staining was carried out using 4′,6-diamidino-2-phenylindole (DAPI) (1:1000) to present profiles of tumor masses, and the merged figures were used to localize the F4/80-positive cells in tumor masses. (b) Some sections were incubated with rat anti-F4/80 antibody and horseradish peroxidase (HRP)–conjugated goat antirat IgG was used as secondary antibody. Bar, 100 μm. (c) Quantification of macrophages in HARA-B tumors. The number of stained cells was counted in five random fields for each of three tumors derived from mice treated with PBS-LIP and Cl_2MDP-LIP at ×200 magnification. Each value represents the mean number of macrophages ± SD. $^*P < 0.05$. (d) Some sections were incubated with rat anti-Gr-1 antibody. Bar, 100 μm. (e) Quantification of neutrophiles in HARA-B tumors. The number of stained cells was counted in five random fields for each of three tumors at ×200 magnification. Each value represents the mean number of neutrophiles ± SD.

Table 3. Histological analysis of inhibitory effects of clodronate-liposomes and reveromycin A on bone metastasis

Treatment	Incidence of metastasis	No. of tumor colonies*		Tumor area (mm^2)*	
		bone	muscle	bone	muscle
PBS-LIP	8/9	4.7 ± 4.1	13.2 ± 14.1	8.1 ± 6.9	16.4 ± 20.2
Cl_2MDP-LIP (200 μl)	5/7	2.0 ± 3.0	7.9 ± 13.1	5.6 ± 9.3	10.1 ± 11.3
Cl_2MDP-LIP (400 μl)	5/9	0.4 ± 0.7†	1.2 ± 1.2†	0.4 ± 0.8†	2.0 ± 3.3
Reveromycin A	6/9	0.7 ± 1.3†	4.3 ± 4.6	3.7 ± 9.2	14.9 ± 13.9

HARA-B cells (2×10^5 per mouse) were injected into the left cardiac ventricle of nude mice on day 0. The mice were s.c. administered PBS-LIP, Cl_2MDP-LIP (200 and 400 μl/mouse once every three days), or reveromycin A (10 mg/kg daily) from day 0 to 6 weeks. The mice were sacrificed at 6 weeks and the formation of metastasis in bone or muscle was examined. The tumor area is represented as the sum of the individual tumor areas in bone or muscle of each mouse calculated as $\pi d^2/4$, where d is the diameter of each tumor in mm. *Mean ± SD, †$P < 0.05$.

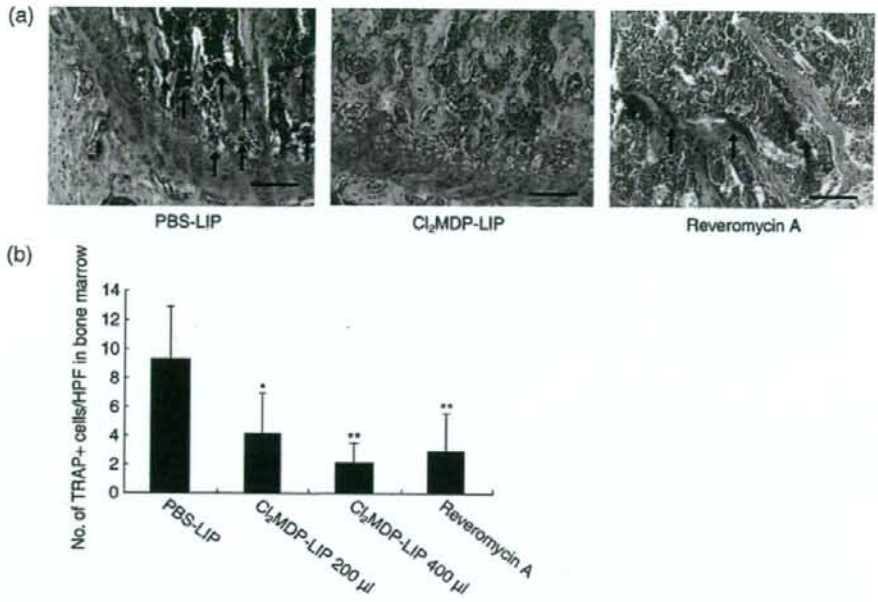

Fig. 5. Reduced number of osteoclasts in bone marrow by clodronate encapsulated by liposomes (Cl_2MDP-LIP). (a) Tartrate-resistant acid phosphatase (TRAP) staining was done using a Sigma Diagnostics Acid Phosphatase kit. The hind limbs of mice treated with PBS-LIP, Cl_2MDP-LIP (200 μL and 400 μL), and reveromycin A in the experiment protocol were taken and fixed in 10% formalin. The specimens were decalcified in a 10% EDTA solution for 1 week and then embedded in paraffin. Arrows indicate TRAP-positive osteoclasts in bone marrow. Bar, 100 μm. (b) Quantification of osteoclasts in bone marrow. The number of TRAP-positive cells in bone marrow was counted under a microscope in five random fields in each of three sections at ×200 magnification. The statistical significance of differences between the controls and other groups was analyzed using the unpaired Student's *t*-test. $*P < 0.05$, $**P < 0.01$.

treated with PBS-LIP revealed numerous macrophages stained with F4/80 in tumors, whereas infiltrating macrophages in tumors were decreased in mice treated with Cl_2MDP-LIP (Fig. 4a). Immunostaining of macrophages also showed a marked decrease in the number of infiltrating macrophages in tumors on treatment with Cl_2MDP-LIP in comparison with the untreated control (Fig. 4b). By contrast, the number of macrophages in bone marrow was not affected by treatment with Cl_2MDP-LIP (data not shown). Quantitative analysis showed a significant decrease of infiltrating macrophages in tumors after treatment with Cl_2MDP-LIP (Fig. 4c). The number of neutrophils in tumors was not affected by treatment with Cl_2MDP-LIP in comparison with the untreated control (Fig. 4d,e).

We further examined whether treatment with Cl_2MDP-LIP at 400 μL as well as reveromycin A affected the number of osteoclasts in bone marrow. The hind limbs of mice treated with PBS-LIP, Cl_2MDP-LIP, and reveromycin A were harvested at 6 weeks after inoculation, and osteoclasts were identified by TRAP staining (Fig. 5a). We observed many TRAP-positive osteoclasts in control mice with bone metastasis. By contrast, the number of osteoclasts was decreased in reveromycin A– and Cl_2MDP-LIP-treated mice. Quantitative analysis demonstrated that the number of osteoclasts was significantly decreased in bone marrow by Cl_2MDP-LIP at 400 μL and by reveromycin A in comparison with the controls (Fig. 5b).

Discussion

In the present study, we assessed the effect of a decreasing number of monocytes/macrophages in peripheral blood and the tumor stroma on bone and muscle metastases using an experimental bone metastasis model in nude mice inoculated with human lung cancer cells which showed strong bone metastasis activity.[3,20] Clodronate-liposomes reduced the number of monocytes in peripheral blood as well as the number of osteoclasts in bone marrow, accompanied by marked inhibition of metastasis to both bone and skeletal muscle by lung cancer cells. Clodronate is a bisphosphonate, and bisphosphonates targeting osteoclast-mediated bone metastasis have been used to treat bone metastasis.[23] The underlying mechanism of their effects is inhibition of a key enzyme in the mevalonate pathway, farnesyl diphosphate synthase, resulting in prevention of protein phenylation and Ras activation, and also producing a unique adenosine triphosphate analog (Apppi), resulting in induction of apoptosis of both osteoclasts and cancer cells.[23] Clodronate encapsulated by liposomes has been developed and successfully applied in several studies for depletion of macrophages.[12,13,21] Although free clodronate is not ingested by macrophages and is rapidly removed from circulation,[24] the liposome-encapsulated form is phagocytosed, and intracellular release of clodronate promotes apoptosis.[21]

Treatment with the osteoclast-targeting agent reveromycin A also significantly decreased bone metastasis by lung cancer cells. Reveromycin A, a novel antibiotic, inhibits bone resorption by inducing the specific apoptosis of activated osteoclasts, possibly because reveromycin A is specifically transported into osteoclasts at acidic pH.[25–27] In this study, histological analyses showed that reveromycin A markedly decreased the number of osteoclasts in bone lesions, suggesting that reveromycin A specifically inhibits osteolytic bone metastasis by targeting osteoclasts in bone lesions. Reveromycin A only slightly inhibited the number of muscle metastases, perhaps through its direct inhibition of cancer cell survival. By contrast, clodronate-liposomes inhibited the survival of macrophages in culture,

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whereas reveromycin A did not. These findings suggest that the clodronate-liposome-induced inhibition of both bone and muscle metastasis may be due to a reduction the number of not only osteoclasts, but also macrophages infiltrating the metastatic lesions in both bone and muscle.

Monocytes in peripheral blood are versatile precursors with the potential to differentiate into the various types of specialized macrophages.[28] Macrophages in the tumor environment are activated by inflammatory responses during the acquisition of malignant characteristics in both the primary tumor and bone metastases.[6] Infiltrating macrophages under conditions of inflammation are derived mainly from peripheral blood monocytes, and create conditions in the tumor stroma and bone metastases that favor metastasis/invasion and angiogenesis through the production of various chemokines, cytokines, growth factors, proteases, and hypoxia.[7–9] Clodronate-liposomes have been shown to significantly reduce the number of monocytes in peripheral blood *in vivo*[12] (see also Fig. 1), and the survival of macrophages *in vitro*. Treatment with clodronate-liposomes also markedly inhibits inflammatory cytokine-induced angiogenesis and infiltration of monocytes/macrophages in the cornea,[12] and also tumor growth by lung cancer cells.[14] Thus, a decrease in the number of macrophages by clodronate-liposomes might also block the metastasis of cancer cells to bone.

Tumor angiogenesis is often closely associated with bone metastasis by cancer cells,[15,29] possibly through angiogenesis in the tumor stroma and also in the metastases themselves. Activation of the *VEGF*, *IL-8*, *bFGF*, and *cyclooxygenase-2* genes in both tumor cells and macrophages in the tumor stroma by inflammatory cytokines induces angiogenesis.[12,30] It has been reported that synergistic interaction between macrophages and tumor cells is required for tumor cell migration through a paracrine loop involving reciprocal signaling of EGF and colony-stimulating factor-1.[31] Inflammatory cytokines produced by macrophages affect tumor invasion and angiogenesis, suggesting that the recruitment of macrophages into the tumor stroma is prerequisite for the acquisition of malignant characteristics.[4–6,9] Previous studies have demonstrated the apparent involvement of macrophages in inflammatory cytokine-induced angiogenesis[12] and also in tumor-induced angiogenesis.[13] The blocking of bone metastasis from lung cancer might be due in part to decreased macrophage-induced angiogenesis. However it still remains to be clarified how bone metastasis is linked to angiogenesis in metastatic lesions.

Treatment with clodronate-liposomes markedly inhibited the metastasis of cancer cells to muscle as well as bone. However, whether a decrease in the number of macrophages by this drug is directly involved in its inhibitory effect on the metastasis to muscle needs to be further studied. Jones *et al.* have recently reported that bone metastasis after the intracardiac injection of melanoma cells is dependent on RANK/RANKL signaling.[32] However, in our present study, the role of RANK/RANKL signaling was not examined. Regarding the pleiotropic mechanisms of bone metastasis by cancer cells, we consider it likely that macrophage lineages provide a microenvironment that is favorable for metastasis and tumor growth not only in bone but also in muscle.[33] The inhibition of metastasis in both bone and muscle by clodronate-liposomes might be due to depletion of osteoclasts precursors as well as tumor-associated macrophages.

In conclusion, osteoclasts are well known to play pivotal roles in bone metastasis by cancer cells, and osteoclasts are derived from monocytes/macrophages. Bisphosphonates are most frequently used to treat bone metastasis in cancer patients, and act by possibly targeting osteoclasts and cancer cells.[34] The present study demonstrated that treatment with a bisphosphonate encapsulated by liposomes markedly decreased both bone and muscle metastases by lung cancer cells. In contrast, treatment with the osteoclast-targeting drug, reveromycin A, specifically inhibited bone metastasis, but not muscle metastasis, by lung cancer cells. These findings suggest that bisphosphonates encapsulated by liposomes may be a novel and potent therapeutic agent against not only bone metastasis but also other organ metastases of lung cancer cells in humans.

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