Applied Radiation and Isotopes 69 (2011) 1713–1716

Contents lists available at ScienceDirect

Applied Radiation and Isotopes

journal homepage: www.elsevier.com/locate/apradiso

Evaluation of BPA uptake in clear cell sarcoma (CCS) *in vitro* and development of an *in vivo* model of CCS for BNCT studies

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ARTICLE INFO

Available online 12 February 2011

Keywords:
p-boron-L-phenylalanine (BPA)
Boron neutron capture therapy (BNCT)
Clear cell sarcoma
Cell line

ABSTRACT

Clear cell sarcoma (CCS), a rare malignant tumor with a predilection for young adults, is of poor prognosis. Recently however, boron neutron capture therapy (BNCT) with the use of *p*-borono-L-phenylalanine (BPA) for malignant melanoma has provided good results. CCS also produces melanin; therefore, the uptake of BPA is the key to the application of BNCT to CCS. We describe, for the first time, the high accumulation of boron in CCS and the CCS tumor-bearing animal model generated for BNCT studies.

1. Introduction

Clear cell sarcoma (CCS) of tendons and aponeuroses is a rare type of melanocytic malignant tumor, accounting for less than 1% of all soft tissue sarcoma. CCS was initially described as a distinct clinicopathologic entity by Enzinger in 1965. Because of the presence of melanin and the positive immunostaining of melanoma-associated antigen HMB-45, Chung and Enzinger (1983) have proposed that CCS be called "melanoma of soft parts". Only CCS, however, not melanoma, demonstrates t(12;22)(q13;q12) translocation that results in chimeric EWS/ATF1 gene expression (Zucman et al., 1993). CCS has a predilection for young adults between 20 and 40 years of age (Enzinger, 1965), with over 95% occurring in the extremities. The standard treatment for CCS is wide surgical resection, and neither chemotherapy nor radiotherapy is effective. Metastasis occurs in more than 50% of such patients, the prognosis of the disease is poor, and the 5-year survival rate is only 54% (Deenik et al., 1999) and 47% (Kawai et al., 2007), with a 10-year rate of 36% (Kawai et al., 2007) and 33% (Lucas et al., 1992). Clearly, new therapeutic methods are required. Recently, boron neutron capture therapy (BNCT) with the use of *p*-borono-L-phenylalanine (BPA) for malignant melanoma has provided good results (Mishima et al., 1989; Mishima, 1997). Since BPA is used in the production of melanin, boron accumulates in tumor cells. CCS also produces melanin; therefore, the uptake of BPA is key to the application of BNCT to CCS. Here, we evaluated the uptake of BPA by CCS cell lines by comparing the specific uptake ability between the mouse melanoma cell line that produces melanin and the more common human sarcoma, malignant fibrous histiocytoma (MFH) cell line. Additionally, a CCS tumor-bearing animal model generated with nude mice was histologically, molecularly and electron microscopically examined for further investigation into the clinical application of BNCT to CCS.

2. Materials and methods

(1) *Boron concentration in CCS*: Human cell lines, MP-CCS-SY (Moritake et al., 2002), SU-CCS-1 (Epstein et al., 1984), KAS (Jishage et al., 2003) and HS-MM (Sonobe et al., 1993) were grown in RPMI 1640 and DMEM (for HS-MM) with fetal bovine serum in a 5% CO_2 humidified incubator at 37 °C. Each CCS cell line was cultured in a culture dish at a density of 5×10^6 for 72 h and exposed to BPA (20, 40, 80 ^{10}B μg/mL) (Stella Pharma Co., Osaka, Japan) in the medium for 4 h. The cells were washed,

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doi:10.1016/j.apradiso.2011.02.006

detached and collected; boron concentration was then measured by ICP-AES (SPS3100, SII NanoTechnology Inc., Chiba, Japan). Each experiment was repeated 5 times.

(2) *Boron concentration in melanoma and MFH cell lines*: Mouse melanoma cell line, B16-F10 (Fidler, 1973) was grown in RPMI 1640, and human MFH cell line Nara-H (Kiyozuka et al., 2001) was grown in DMEM with fetal bovine serum in a 5% CO_2 humidified incubator at 37 °C. Each cell line was cultured and exposed to BPA (80 ^{10}B μg/mL) in the medium (80 ppm) for 4 h. The cells were washed, detached, collected and measured by ICP-AES in the same way as the CCS cell lines. B16-F10 was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Each experiment was repeated 5 times.

(3) *Tumor-bearing animal model of CCS*: All animal experiments were carried out according to the regulations of the Animal Care and Use Committee of Hyogo Cancer Center. Cells (10^7) of cell line MP-CCS-SY were subcutaneously transplanted into the back of each BALB/c AJcl-nu/nu nude mouse (CLEA Japan Inc., Tokyo, Japan). Four weeks thereafter, the animal was killed and the tumor mass was resected.

(4) *Histological and molecular examination of tumor resected from tumor-bearing animal model*: Tissue samples were fixed in 10% buffered-formalin solution. All specimens were paraffin-embedded according to standard protocols; 4-μm-thick sections were then stained with HE and Fontana-Masson. Immunostaining was carried out with antibodies HMB-45 (Dako Japan, Tokyo, Japan; diluted 1:75) and Melan-A (Nichirei, Tokyo, Japan; diluted 1:10) for testing the expression of melanoma-associated antigens in tissue samples, and with S-100 (Dako Japan; diluted 1:2000). The HMB-45 antigen localized primarily in premelanosomes, while the Melan-A antigen localized in pigmented melanosomes. The Fontana-Masson stain was used for the detection of melanin pigments. For immunostaining with Melan-A, the deparaffinized tissue sections were heated in 10 mmol/L citrate buffer (pH 6.0) for 60 min at 95 °C; for HMB-45, proteinase K (Dako Japan) was used for proteolytic digestion for 4 min at room temperature. Subsequently the sections were routinely processed by the automatic Ventana BenchMark XT immunostaining system (Roche Diagnostics Japan, Tokyo, Japan). Furthermore, the molecular genetic technique was carried out for the detection of the chimeric EWS/ATF1 gene (for the diagnosis of clear cell sarcoma) by reverse transcription polymerase chain reaction (RT-PCR), as described (Zucman et al., 1993; Moritake et al., 2002).

(5) *Electron microscopy*: The samples were fixed for 2 h in 1% glutaraldehyde, 4% buffered formalin, postfixed in 1% osmium tetroxide, and embedded in epoxy resin under standard procedures. Ultrathin sections were cut and double-stained with uranyl acetate and lead citrate and examined through an electron microscope (JEM-100S/SX, JEOL Ltd., Tokyo, Japan).

2.1. Statistical analysis

Results are expressed as the means ± SD (Standard deviation of the mean). Statistical analysis was carried out with the JMP® software (SAS Institute Japan, Tokyo, Japan). Assay results were analyzed for differences with the use of one-way analysis of variance (ANOVA) followed by the Tukey-Kramer honestly significantly difference test, to determine significant differences, if any, among the groups. Means were considered statistically different at $p < 0.05$.

3. Results

The proliferation of CCS, melanoma and MFH cell lines was not affected by BPA at up to 200 μg of ^{10}B/mL in the medium (data not shown). The four CCS cell lines took up the boron in a concentration-dependent manner. In the presence of 20, 40 and 80 ppm of ^{10}B in the medium, the concentration of boron in HS-MM was significantly lower than in the other three cell lines; in the presence of 80 ppm, the uptake of boron by MP-CCS-SY and SU-CCS-1 was almost two times the uptake by HS-MM, demonstrating a disparity of boron uptake ability among these four CCS cell lines (Fig. 1). In a comparison of the BPA uptake ability between melanoma (B16-F10) and MFH (Nara-H) cell lines on the one hand, and MP-CCS-SY and HS-MM lines on the other, MP-CCS-SY demonstrated more than 2 times the uptake compared with that of B16-F10 and Nara-H. Although HS-MM showed the lowest uptake among the CCS cell lines, statistically it demonstrated almost the same uptake ability as that of B16-F10. Furthermore, the uptake by Nara-H was lower than that by HS-MM and almost the same as that by B16-F10 (Table 1). Since the MP-CCS-SY cell line showed the highest BPA uptake at 80 ppm, it was selected for generating the tumor-bearing animal model, and it produced a solid tumor mass under the skin 4 weeks after the implantation (Fig. 2a). The tumor adhered firmly to the surrounding soft tissue in the subcutaneous space, and a very large feeding artery was observed (Fig. 2b). The resected solid tumor mass was almost 1 cm in diameter (Fig. 2c), its cross-section was slightly viscous and it bled easily (Fig. 2d). Microscopically, CCS was composed of nests of monotonous polygonal cells with clear cytoplasm and no melanin pigment upon HE (Fig. 3a and b), or Fontana-Masson staining (Fig. 3c). Immunohistochemically, each cell line was positive for Melan-A, HMB-45 and S-100 (Fig. 3d–f). Also, the EWS/ATF1 fusion gene was detected by RT-PCR in the tissue of the CCS tumor-bearing animal model (data not shown). Electron microscopic examination of the tissue from the CCS tumor-bearing animal model showed the expression of few melanosomes in cytoplasm, although no melanin was detected on either HE or Fontana-Masson staining (Fig. 4).

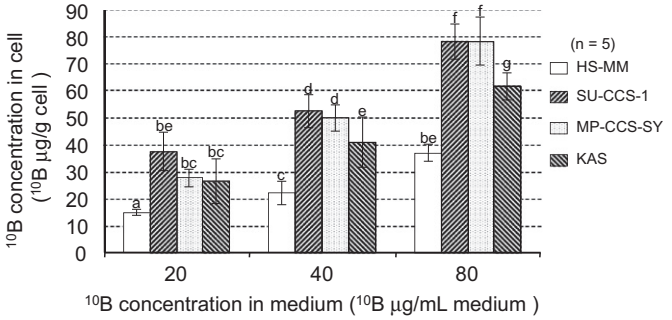

Fig. 1. Four human CCS cell lines (HS-MM, MP-CCS-SY, SU-CCS-1, KAS) were tested for their uptake of boron. Each cell line took up the boron in a concentration-dependent manner. In the presence of 80 μg of ^{10}B/mL in the medium, the uptake by SU-CCS-1 and MP-CCS-SY was almost two times that by HS-MM. Pooled results are presented as the means ± SD of five independent experiments, and significant differences are indicated by different letters ($p < 0.05$).

Table 1
Comparison of the uptake ability of BPA by melanoma (B16-F10), MFH (Nara-H) and CCS (MP-CCS-SY and HS-MM) cell lines. The uptake by MP-CCS-SY was more than 2 times that by each melanoma or MFH cell line; the uptake by the HS-MM cell line was almost equal to that by the melanoma cell line. Statistically, the uptake by B16-F10 and Nara-H was almost the same. Values that are statistically different from one another ($p < 0.05$) are indicated by different letters.

Cell Line (n=5)	Means ± SD (^{10}B μg/g cell)
MP-CCS-SY	[a]78.3 ± 8.8
HS-MM	[b]37.1 ± 2.9
B16-F10	[bc]29.4 ± 1.5
Nara-H	[c]27.4 ± 3.1