Efficacy of boron neutron capture therapy on liver metastases of colon adenocarcinoma: Optical and ultrastructural study in the rat

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Abstract. The effect of neutron boron capture therapy (BNCT) was studied in rat tumor liver cells after induction of the liver metastases by splenic inoculation of cells from DHA/K12/TRb line. Ten days following the treatment, the BPA was injected into rats and therefore the animals were sacrificed, the liver was exposed to neutron irradiation and processed. In some experiments the liver was reimplanted (after irradiation) in syngenic animals and studied 3 days later, following sacrifice. Samples of tissue obtained from metastatised and non-metastatised areas of the liver parenchyma, before and after the neutron irradiation, were examined in light microscopy and electron microscopy. The analysis pointed out damages induced by the neutron treatment in single tumor cells mostly localised in the synusoidal blood stream. Debris and apoptotic cells were sometimes observed in the neoplastic nodules before treatment, while the tumor cell death (apoptosis) increased in the tumor cells following BNCT treatment. An intense scavenger activity of Kupffer cells after irradiation was accompanied by a strong acid phosphatase reaction detectable in wide cytoplasmic areas. In the liver parenchyma of reimplanted animals, the presence of large collagen bundles spread among the hepatocytes was observed at electron microscopy.

Introduction

Liver metastases from colon carcinoma remain a major cause of morbidity and mortality in the human population despite advances in diagnosis and treatment. When metastases are diffused to both lobes, they are not amenable to surgical resection, and are usually resistant to chemotherapy. This therapeutic defeat is particularly disappointing when considering that in most cases the primary tumor in the colon can be excised easily and liver is often the only site of metastatic spread. For these cases of inoperable diffused liver metastases (30%), we planned a new procedure based on the application of boron neutron capture therapy (BNCT) This therapy appears to be very promising in the selective treatment of liver metastases being based on the ionising effect of the α particles released in the nuclear reaction between 10B and thermal neutrons (1). Starting from 1986, the multidisciplinary ‘Taormina Project’ (2-4), has involved physicians, biologists, chemists, nuclear physicists and surgeons. The therapeutic effect of BNCT on diffuse hepatic tumors has been performed, after tumor enrichment with 10B, irradiating isolated liver with a slow neutron beam inside the thermal column of a nuclear reactor, which is available in the Department of Nuclear and Theoretical Physics of the University of Pavia, Italy. The study has just been articulated in three phases: i) selection of a drug as 10Boron carrier, with organ and tissue specificity (5); ii) assessment of biodistribution of boron in the experimental model of liver metastases in the rat and iii) evaluation of the therapeutic effect of BNCT in vitro (in cell cultures) and in vivo (in an animal model). In order to verify the clinical feasibility and the biological effectiveness of the treatment in patients, it is crucial to develop experimental models in vivo capable of selectively increasing 10B concentration in tumor cells.

We reported a morphological (light and electron microscopy) and histochemical approach to study the cytotoxic effect of BNCT on the rat liver bearing colon adenocarcinoma metastases. The observations were performed before and after the irradiation of the organ in extra situm and, in some cases, following the reimplantation of the organ. Particular attention was paid to this later situation when the physiology and the metabolism of the liver was reactivated.

Materials and methods

Cell line. DHD/K12/TRb cell line has been established from a rat colon carcinoma chemically induced (1,2 dimethyl-
(hydrazine) in a BD-IX strain rats. This line was selected and cloned for its capacity to induce progressive and metastatic tumors in the syngeneic host (6). Cells were cultivated as monolayer in 25 cm² tissue culture flasks in Ham's F10 medium supplemented with 10% foetal calf serum (complete medium). Cell viability was assayed by Trypan blue exclusion and was always found to be above 90%.

**Animals.** Six-week old BDIX male rats weighing 300 g were obtained from Charles River, Italy and were kept under pathogen-free conditions. Tumor cell injection was performed under general anesthesia and the rats were harvested in standard conditions with food and water ad libitum.

**Induction of liver metastases.** Colon carcinoma metastases were induced (7,8) in rat liver by splenic inoculation of 2x10⁷ stabilised cells (DHD/K12/TRb). To prevent tumor induction in two hepatic lobes, we clamped one peripheral portal vessel during the splenic injection of tumor cells. The healthy parenchyma was used as a control. Diffused hepatic micrometastases were observed after 48 h of the splenic inoculation and many macrometastases after 10 days.

**Study of biodistribution of BPA.** Ten days following the tumor cell inoculation, the boron compound solution was intravenously injected into the rat (300 mg of BPA/kg). Three hours after the boron administration, the animals were sacrificed and the liver was washed by perfusion of a glucose solution. Then the liver was stored at 4°C during the irradiation (10 min) by thermal neutrons at the position inside the thermal column of the Triga Mark II Reactor of the University of Pavia. Each sample was given a neutron fluence of 10¹³ cm⁻². In some experiments, the irradiated liver was reimplanted in syngeneic rats and observed 3 days later.

**Light microscopy.** Smears obtained from cell cultures of DHD/K12/TRb cell line were stained with May Grunwald-Giemsa and paraffin sections obtained from normal and tumoral liver areas were stained with hematoxylin and eosin for morphology studies. On cryostatic sections (6 µm thick) obtained with a Leica CM 1850, the acid phosphatase reaction was carried out. The samples were incubated using an azo-coupling method: 18% PVA in 100 mM sodium acetate buffer pH 5.0; 10 mM hexarotized-p-rosanilin prepared immediately before the use from basic fucsine (BDH, Poole, England) and 5 mM naphthol AS-BI phosphate acid (Sigma Chemical Co). The incubation was carried out for 30 min at 37°C. The samples were washed in distilled water and mounted in glycerin-gelatin.

In order to visualize the acid phosphatase reaction in gray levels, the difference between the positive area (originally red) respect of the surrounding one (originally light brown), the image was digitalized with a flatbed scanner at 600 dpi, converted in gray levels, the positive area was submitted to an enhancement with the image analysis software Media Cybernetics ImagePro Plus 4.5 and then superimposed.

**Results and Discussion**

Fig. 1 shows the experimental model for inducing the colon cancer metastases in the liver utilised in our study. The experiment is articulated in 4 steps: i) inoculation of the cancer cells to induce the metastases; ii) assessment of biodistribution of boron in the liver; iii) neutronic irradiation of the explanted liver and histological analysis and iv) evaluation of the therapeutic effect of BNCT in the liver examined 3 days after the reimplantation. The colon carcinoma cell line utilised was heterogeneous in size (Fig. 2A) and in cell ploidy (data not showed). Single tumor cells were found spread in the lumen of the sinuoids or organised as metastatic nodules in the parenchyma (Fig. 2B), sometimes organised in pseudo-glandular structures similar to colon tubular glands. Often the single and clustered tumor cells appeared in differentiated state because of the presence in their cytoplasm of storage material resembling secretory granules (Fig. 3A). The liver metastases were surrounded by
Figure 2. A, Colon cancer cells (DHD/K12/TRb) in culture showing heterogeneous size. May Grunwald Giemsa stain (x1200); B, Paraffin section from liver tumor area with neoplastic nodules (arrows). Haematoxylin and eosin stain (x1200).

Figure 3. Ultrastructure (A-C) and light microscopy (D) of the liver before (A) and after (B,C,D) BNCT treatment. A, A metastatic cell showing electron-dense bodies in the cytoplasm (x4000); B and D, After irradiation apoptotic events (asterisk and arrow) are frequent as a consequence of the damage (x4000, x800); C, Kupffer cell with a large haeterophagic vacuole containing an apoptotic body (arrow) (x4000).
a fibroinflammatory corona, composed of mononuclear inflammatory cells such as monocytes, lymphocytes, pit cells and plasma cells (9). A different degree of Kupffer cell phenotype was observed.

The rats sacrificed 10 days after tumor inoculation and BNCT treatment, showed morphological alterations detectable both at light and electron microscopy in both the free tumoral cells and the aggregation of neoplastic cells in nodules. Some neoplastic cells showed a different degree of damage up to cell death. The cell degeneration often showed the morphological characteristics of apoptosis (Fig. 3B and D). As a consequence of this experimental situation (after irradiation), an increased number of activated Kupffer cells showing heterogeneous morphophenotype was observed. The scavenger activity of the Kupffer cells was mainly addressed to the uptake and digestion of cell debris and apoptotic bodies probably derived from damaged tumor cells (Figs. 3C and 4B). A strong phosphatase reaction (as expression of intense lysosome activity) was always observed in these cells underlying their activation state (Fig. 4A) (10). The tumor cells organised into cords or lobules observed before BNCT treatment did not show evident signs of damage or degeneration. On the contrary, after treatment: in the nodules some tumoral cells appeared in degeneration phases sometimes with apoptotic signs. In a series of three experiments of liver reimplantation in syngeneic rats, a microsurgical technique modified according to Kamada method (11,12) was utilised. The whole BNCT procedure (13) was applied with reimplantation of irradiated liver into syngeneic rats. After a three-day period of survival reimplanted monodispersed cells demonstrated a higher expression to radiation damage with various aspects of nuclear and/or cytoplasmic sufference up to cell death by apoptosis. Similar aspects were also observed in the external and internal cell layers of tumoral nodules, supporting the fact that boron assumption and radiation damage are not limited to the periphery of neoplastic clups. No apoptotic signs were present in healthy liver parenchymal cells. In accordance with these data, increased phosphatase activity was observed in Kupffer cells showing strong cell

Figure 4. Kupffer cell activation after BNCT treatment. A, Light microscopy (computer-enhanced picture) of a strong acid phosphatase activity in a Kupffer cell observed in a cryostat liver section (x1200); B, Ultrastucture of a Kupffer cell (large arrow) in phagocytosis and degradation phase of an engulfed cell body (thin arrow) (x4000).

Figure 5. Ultrastructure of an evident stromal reactivity in a reimplanted liver after BNCT treatment: presence of collagen bundles spread (arrows) in the parenchyma (x4000).
fragmentation. The identification of collagen bundles (Fig. 5) only in the liver parenchyma of reimplanted animals evidence the efficacy of the BNCT treatment.

References