Acta Oncologica Vol. 34, No. 3, pp. 335–338, 1995

ENERGY STATUS PARAMETERS, HYPOXIA FRACTION AND RADIOCURABILITY ACROSS TUMOR TYPES

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Under full nutrient in vitro conditions, the cellular adenylate energy charge of six different rodent and human tumor cell types was identical, i.e., 0.94 ± 0.01, suggesting the potential utility of this parameter as a cell (and tissue) independent marker of nutrient deprivation and hypoxia, across tumor types. The adenylate energy charge values of tumors, arising from these cells, was reduced and variable ranging from 0.72 to 0.91 for the various tumor types. However, neither the tumor adenylate energy charge, NTP/Pi, nor PCr/Pi ratios correlated with the radiobiologic hypoxic cell fractions across tumor types. The reduced adenylate energy charge in vivo suggests varying degrees of nutrient deprivation in the different tumor types, however, factors other than or in addition to hypoxia likely contribute to tumor energy status.

Studies employing experimental rodent and xenografted human tumors have shown that with few exceptions, for a given tumor type, naturally occurring or induced decreases in tumor blood flow result in a decreased tumor energy status (1–4). The flow limited nutrient considered most likely to elicit a decrease in energy status is oxygen. Reduced oxygen availability and a reduction in the oxidative pathway of energy generation likely lead to an imbalance between the generation and utilization of high-energy phosphates, and a decrease in energy status. This interpretation has been strengthened by studies demonstrating that in mice bearing the spontaneous murine tumor MCaIV, an increase or decrease in the inspired oxygen concentration elicited an increase or decrease in the NTP/P_i or PCr/P_i ratios, evaluated by ^{31}P magnetic resonance spectroscopy (MRS) (5). Additionally, naturally occurring changes in the hypoxic fraction, (i.e., with tumor growth) of the murine tumor FSaII have been shown to correlate with decreasing energy status (6) and in separate studies with tissue pO_2(7). These studies demonstrate a role for oxygen in the maintenance of energy status, and have given rise to the hope that the non-invasive assessment of tumor energy status by MRS may be useful for non-invasively assessing tumor hypoxia, a condition which markedly reduces the efficacy of radiation and some chemotherapeutics.

The relationship between energy status, and hypoxic fraction across tumor types has received substantially less attention. Rofstad et al. (3) observed no relationship between the MRS derived ratio (NTP + PCr)/P_i and the radiobiologic hypoxic fraction in two transplanted rodent and two xenotransplanted human tumors. In the murine tumors FSaII and MCaIV, no correlation was observed between the radiobiologic hypoxic fraction and the NTP/P_i or PCr/P_i ratios (2). This lack of correlation may be due to naturally occurring endogenous differences in the levels of PCr, NTP, or P_i between tissues. ^{31}P-MRS evaluation of various normal tissues reveals substantial variation in the ratio of high to low energy phosphates. Tissue specificity of ^{31}P-MRS ratios would preclude this approach for evaluation of hypoxia across tumor types. A potentially promising parameter of energy status is the adenylate energy charge, i.e., ([ATP] + 1/2[ADP])/([ATP] + [ADP] + [AMP]). This is a tightly regulated parameter of energy

Received 14 October 1994.
Accepted 13 December 1994.
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Paper presented at the Tumour Microenvironment Workshop in Granada, Spain, September 23–25, 1994.

 ISSN 0284-186X

status, and previous studies indicate that the value of this parameter is relatively invariant in various tissues (8, 9).

In the present study, the relationship between tumor radiobiologic hypoxia, the 50% tumor control dose, and energy status (MRS and adenylate energy charge), has been examined across tumor types in xenografted human and isografted rodent tumors. These studies concern whether adenylate energy charge (and MRS) parameters of tumor energy status is sufficiently closely linked to tumor hypoxia to be of value in assessing hypoxia across tumor types.

Material and Methods

Animals, tumors, and cells. Tumors used in the present study included four which spontaneously arose in C3Hf/Sed mice: the mammary carcinoma, MCaIV; fibrosarcoma, FSaII; squamous cell carcinoma, SCC VII; and an undifferentiated sarcoma, Sa1. The human cell lines, FaDu, a pharyngeal squamous cell carcinoma and U87, a high-grade glioma, were obtained from American Type Tissue Culture (ATTC), Rockville, Maryland. Rodent tumor cells were isotransplanted in C3Hf/Sed mice and the U87 and FaDu cells xenotransplanted in NCr *nu/nu* mice. For all in vivo studies, third to fifth generation tumors were initiated in 6–8-week-old male and female mice.

Cells were cultured in minimum essential medium (MEM) containing Earle's balanced salt solution ($\approx$ pH 7.4 in 5% $C0_2$), and 12% heat inactivated fetal calf serum. FaDu and U87 cells, used to initiate source tumors, were studied over the subsequent 5–12 in vitro passages. Rodent cells were cultured through approximately five passages, and used for survival curve determinations between the fifth and twelfth passage. The detailed procedures and results of the cellular radiosensitivity and tumor control dose assays have been previously published and are described here only briefly (10).

In vivo assays. Tumors were initiated from single cell suspensions of excised source tumors injected into the subcutaneous tissue of the lower limb. Nude mice were given 5.6 Gy whole-body irradiation 24 h prior to transplantation. The mice were randomized into treatment groups and radiation dose levels. Six to eight animals were injected per dose level. Tumors were irradiated at an average tumor diameter of 6–6.5 mm. Irradiations were performed with 3 cm parallel opposed ^{137}Cs fields. Prior to irradiation, (as well as MRS, tumor excision, and energy analysis), all animals were anesthetized with 0.04 mg sodium pentobarbital g^{-1} body weight. Prior to irradiations under local hypoxia, a spring loaded brass clamp was applied to the base of the thigh. Following irradiation, the animals were examined and scored 3–5 times per week for tumor shrinkage, recurrence, and death. Upon reaching approximately 8 mm diameter, the animal was scored as a recurrence and killed. Non-recurrent animals were examined for 120–180 days. Following termination of the experiment, the data were fitted to a logit regression line and the TCD_{50} values and confidence intervals were calculated, as described previously (10).

In vitro radiosensitivity. In vitro survival curve analyses were performed with emphasis placed on the relatively high-dose, low surviving fraction response. Irradiations were performed at the time cells actively entered the growth phase, to minimize the effects of trypsin-induced radiosensitization. Initial (plating efficiency) multiplicities ranged from 1.04 to 1.15, and multiplicities at the time of irradiation ranged from 1.4 to 2.2. Cells were irradiated from 24 to 40 h after plating, to doses ranging from 0 to 12 or 14 Gy in 2 Gy increments. Following irradiation, the various cell lines were incubated for approximately 10 to 16 days for colony formation, depending on the dose administered and the cells' proliferative rate. The cellular surviving fractions were calculated as the ratio of the number of colonies to the number of colony forming units in the irradiated versus control (PE) cells, after correcting for initial and final multiplicity.

Energy analysis. Analyses of tumor energy status were performed on 6–6.5 mm average diameter tumors. These studies were performed concurrently with the TCD_{50} studies, ^{31}P-MRS studies, and the HPLC analyses for ATP, ADP, and AMP, i.e., from the group of tumors arising from the same tumor cell transplantation. For the MRS studies, the spectrometer used and procedures employed are as previously published (6). Briefly, the tumor bearing foot of anesthetized mice was secured with surgical thread tied to the digit of the tumor bearing foot. Spectra were collected on a wide bore system (General Electric NT-300) operating at 121 MHz for phosphorous. Solenoid coils of four turns approximately 1.2 cm in length were employed. Spectral accumulations used a 60° tip angle, a pulse interval of 2.1 s, a spectral width of $\pm$ 12 000 Hz, and 512 free induction decays. The B_0 field was shimmed on the in vivo ^1H water signal of the tumor prior to spectral accumulation. Spectral processing included 25 Hz of exponential multiplication to reduce high frequency noise. Peak areas were measured by fitting the spectrum to a series of Lorentzian peaks provided by the manufacturer. No attempts were made to correct for the effects of partial saturation. Tumor and cell extracts were analyzed for adenylate phosphates by HPLC using previously described procedures (2). The protruding superficial tumors were excised from anesthetized animals with surgical scissors and immediately frozen in liquid nitrogen. The frozen tissue was extracted with 0.4 M perchloric acid at 0°C. Similarly, sub-confluent monolayers of cultured tumor cells in 30 cm^2 flasks were frozen by submersion of the flask in liquid nitrogen. Following the addition of perchloric acid and extraction, the cell extracts were centrifuged and supernatant neutralized, all at 0–5°C. The neutralized

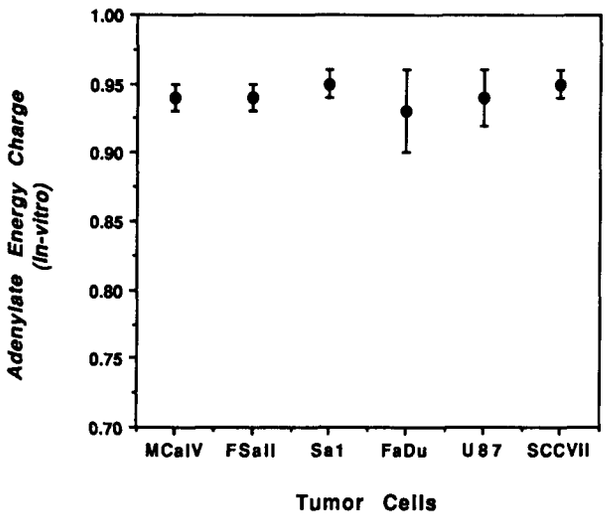

Fig. 1. The mean and standard deviation of the adenylate energy charge of the indicated cell lines obtained under full-nutrient oxygenated conditions in vitro.

extracts were separated on a strong ion exchange column and quantitated at 254 nanometers.

Results

The adenylate energy charge of the various tumor cells studied under defined in vitro conditions is shown in Fig. 1. The energy charge (ATP + 0.5ADP)/(ATP + ADP + AMP) is uniformly high and similar in the various tumor cell types under aerobic, exponential growth phase conditions. These results suggest than the tumor cell type does not influence the value of this energy status parameter.

Fig. 2 shows the relationship between the various parameters of energy status and the radiobiologic tumor hypoxic fraction. An unanticipated number of deaths due to metastases precluded the determination of the hypoxic fraction in the SCCVII tumors and these data are not included. Four of the five evaluable tumor models contained hypoxic fractions in the 1 to 10% range, with the remaining tumor (MCaIV) exhibiting a hypoxic fraction of approximately 70%. For these 5 tumors, significant differences in the adenylate energy charge did not correlate with the hypoxic cell fraction. The MCaIV tumor which contained a relatively large hypoxic fraction exhibited an energy charge value similar to tumors with hypoxic fractions in the 2 to 10% range. A similar lack of correlation was observed for the MRS derived parameter NTP/P_i. The PCr/P_i parameters were not resolvably different with the exception of the value obtained for the MCaIV tumor where its relatively large value was observed in the most hypoxic tumor. Although none of the energy status paramaters exhibited differences which could be correlated with tumor hypoxia, the energy charge values were substantially lower than under full-nutrient in vitro conditions, suggesting a substantial degree of nutrient deprivation in these tumor models.

Discussion

The similarity of the adenylate energy charge among the various tumor cell lines under full nutrient conditions, clearly demonstrates the lack of tissue type specificity for this parameter and suggests its utility for assessing nutrient dependent energy status. However under in vivo conditions, the tumor adenylate energy charge is significantly reduced (in 4 of 5 tumors), but no correlation between the

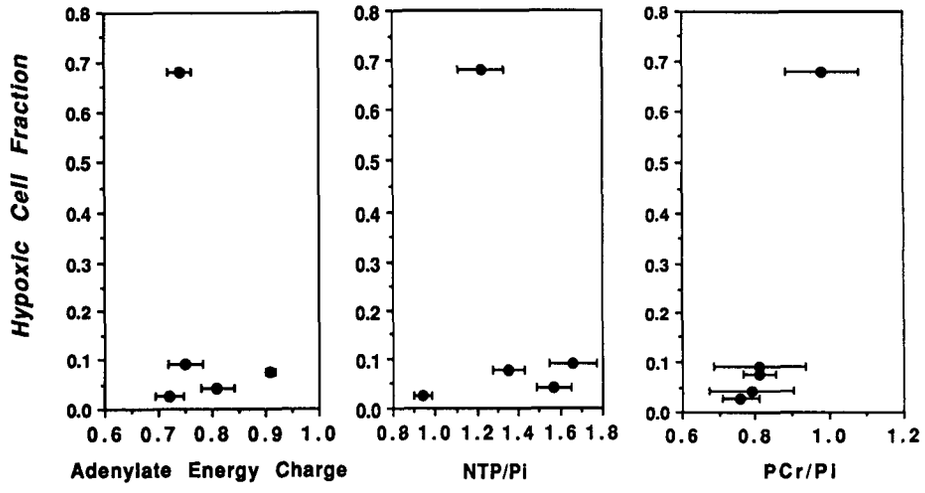

Fig. 2. The radiobiologic hypoxic fraction of various tumor types plotted as a function of three parameters of tumor energy status. The tumor types are as indicated in Fig. 1 with the exception of SCCVII. Tumors of a particular type were initiated from the same stock tumor cell suspension and were randomized to the various analysis groups, e.g. hypoxic cell fraction or various energy status parameters. Parts of these data (hypoxic fractions) have been previously published (10). Error bars are one standard deviation.

radiobiologic hypoxic fraction and the energy charge is observed.

Two factors which could explain the lack of correlation between energy status and radiobiologic hypoxia in vivo are: 1) a difference in the oxygen concentration which gives rise to radiobiologic hypoxia versus an inhibition of oxidative phosphorylation, and 2) involvement of an alternate pathway of energy generation, i.e., glycolysis. Studies conducted in CHO cells in the presence and absence of glucose, have shown that in the presence of glucose, severe oxygen deprivation minimally impacts cell energy status (11). In the absence of glucose, rapid decreases in energy charge occur over the oxygen concentration range which alters radiation sensitivity. Furthermore, glucose deprivation alone (in the presence of oxygen) substantially reduced cellular ATP. Similar conclusions are suggested from studies comparing the adenylate energy charge of murine FSaII and MCaIV tumor under conditions of severe oxygen deprivation plus glucose (2). The adenylate energy charge decreases approximately three times more rapidly in the FSaII tumor cells in the absence of oxygen.

In summary, neither the MRS derived parameters of tumor energy status, nor the tumor adenylate energy charge correlates with the radiobiologic hypoxic fraction across tumor types. The cell and tissue specificity of some energy status parameters, and non-oxidative pathway for energy production, precludes a determination of whether oxygen and/or glucose is the cause of decreased energy status on an a priori basis and, therefore, quantitation of the tumor hypoxic fraction from energy status parameters.

ACKNOWLEDGEMENTS

This work was supported by NIH grant No. CA-22860.

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