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Mapping and quantification of biomolecules in tumor biopsies using bioluminescence

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Abstract. Quantitative bioluminescence and single-photon imaging have been applied for mapping concentration distributions of metabolites, such as adenosine triphosphate (ATP), glucose and lactate, in biopsies of cervical cancers in patients. Biopsies were taken before a conventional radiation treatment, and a number of clinically relevant data, such as local tumor control, patient survival, metastatic spread and so forth, were documented. There was no correlation between staging or grading and any of the metabolic parameters measured. Local correlations between ATP, glucose and lactate on a pixel-to-pixel basis were generally positive, with respective Spearman's correlation coefficients less in patients without clinically documented metastasis compared with those with metastatic spread. Lactate concentrations were significantly higher and scattered over a wider range in tumors with metastatic spread in comparison to malignancies in patients without metastasis. Thus, high local lactate levels of ≥ 20 μmole/g appear to be associated with a high risk of metastasis, at least in human cervical tumors.
Key words. Metabolic imaging; bioluminescence; single-photon imaging; human tumor biopsies.

Introduction

The biological behavior of tumor cells in vivo is determined not only by intrinsic genetically determined properties but also by the microenvironment of cells in malignant tissues[1-4]. This is true for growth characteristics of solid tumors and their aggressiveness towards the tumor host. Also, the susceptibility of tumor cells to certain therapies can be largely modified by factors in the micromilieu of cancers[3].

The various organs in a healthy organism maintain a metabolic milieu which matches the requirements of every cell. This is achieved by regulating the distribution of blood perfusion between different organs or between different regions within one organ. Such a regulation is carried out by the autonomous nervous system, by intrinsic properties of vascular smooth muscles, by hormones and mediators, as well as by local metabolic influences[1].

In most solid tumors, severe disturbances of the microcirculation occur already at an early state of growth. The abnormalities of the tumor vasculature include a loss of the natural hierarchy of blood vessels, changes in the normal vascular density and loss of the physiological regulation of blood perfusion, as well as the emergence of pronounced spatial and temporal heterogeneities in the blood supply[6,7]. As a consequence, solid tumors often exhibit regions with insufficient blood perfusion adjacent to areas with luxurious blood supply. Relevant factors that modulate tumor growth and therapeutic sensitivity, such as local oxygen pressure, concentrations of metabolites and waste products, may thus vary largely within malignancies[4,13]. Such heterogeneities in the metabolic milieu have been demonstrated for experimental tumors as well as malignant diseases in patients[2,5]. The significance of tissue oxygen pressure as a predictive parameter has been clearly shown for cervical cancer in patients in a systematic clinical study[5].

The present study was undertaken to further investigate the metabolic milieu in human cervical tumors. Investigations were carried out on tumor biopsies that were routinely taken for pathohistological classification of the malignancies at the Radium Hospital in Oslo. The biopsies were rapidly frozen in liquid nitrogen and transferred on dry ice to our laboratory, where metabolic measurements were made. To characterize metabolites in these human biopsies, a technique using quantitative bioluminescence and single-photon imaging was applied[10-12,16].

Materials and methods

A total of 12 biopsies from 10 patients were obtained for measurement. The biopsies were taken from patients in stage 2 or stage 3 (Fédération Internationale de Gynécologie et d'Obstétrique) of the disease and were randomized with regard to the anatomical site of removal. Biopsies were removed from the tumors before conventional radiation treatment. Relevant patient data, such as tumor shrinkage, patients' survival, incidence of metastasis and so forth, were documented. For metabolic imaging, cryostat sections were made from the frozen tumors that adhered to the upper side of a cover glass. The cover glass was laid upside down on a glass slide with a regtancular casting mold. The mold was filled with a frozen enzyme solution containing

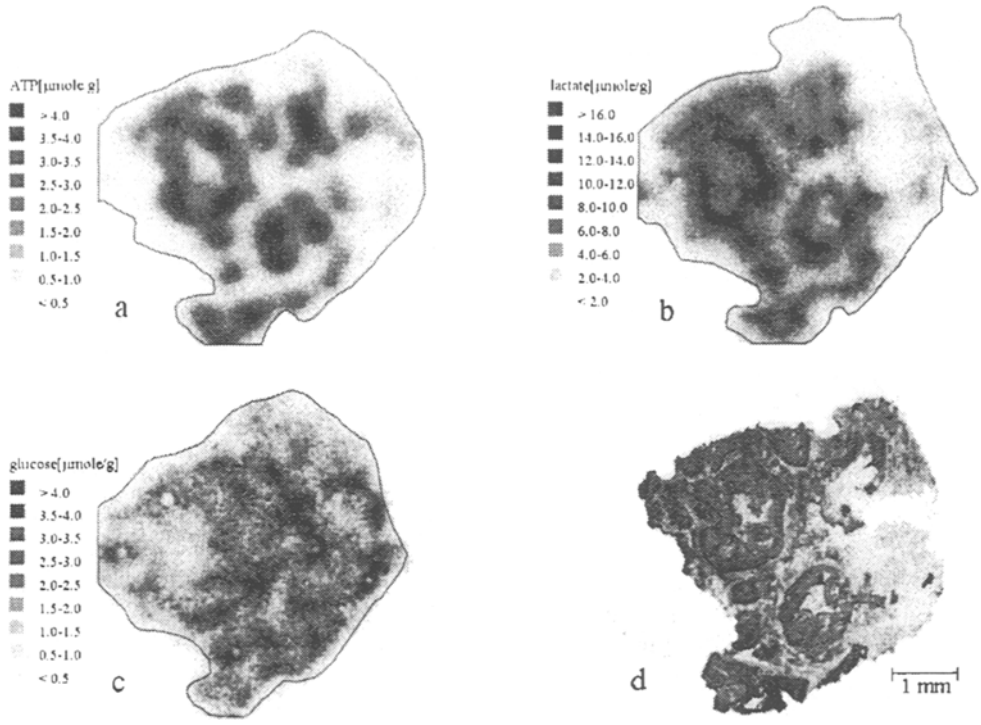

Figure 1. Metabolite distributions (coded with different gray levels) in a human cervical tumor. For comparison, a histological section is shown in the lower right panel (d).

luciferase and various other enzymes to link the substrate of interest to the luciferase light reaction. Different mixtures of enzymes and luciferases were used to detect ATP, glucose and lactate. However, these enzyme solutions were applied to serial sections to determine the different metabolites at quasi-identical locations within the biopsies.

The whole array was kept frozen until it was transferred to a microscope stage in an air-conditioned environment. Thus, the temperature of the array was raised above the melting point of the tissue section and of the frozen enzyme solution which was associated with the initiation of the luciferase reaction and light emission. The temperature-controlled environment guaranteed reproducible kinetics of the enzyme reactions. The spatial distribution of the bioluminescence intensity within the tissue section was registered directly using an appropriate microscope (Axiophot, Zeiss, Oberkochen, Germany) and an imaging photon-counting system (Argus 100, Hamamatsu, Herrsching, Germany).

Photon intensity was calibrated with reference to appropriate standards to obtain density distributions of ATP, glucose and lactate in absolute volume-related tissue concentrations (micromoles per gram wet weight). These values were routinely validated by independent measurements with high-performance liquid chromatography (HPLC) and enzymatic standard assays, respectively.

The digitized images of the different substrate distributions as well as of an adjacent tissue section stained with haemotoxilin and eosine were transferred to a personal computer with commercial imaging software. By optical overlay of the metabolite distributions with the histological section, metabolites were evaluated in tumor regions with densely packed viable cancer cells assigned 'neoplasia', in areas with necrosis and stromal tissue elements termed 'necrosis' and eventually in surrounding normal tissue. Furthermore, a computer algorithm allowed for the pixel-to-pixel correlation among the images of the different substrates[8,15]. Further details on the technique of bioluminescence in imaging photon counting have been published elsewhere[11,16].

Results

Metabolite distributions in all the tumors investigated were extremely heterogeneous. Figure 1 presents distributions of ATP, glucose and lactate and a serial section stained for histological observation. It clearly shows a positive correlation between ATP and lactate and potentially between ATP and glucose. An equivalent distribution pattern can also be recognized in the histological structure of this tumor.

Drastic concentration differences were obvious preferentially between vital and necrotic tumor regions. However, the interindividual differences were even more pronounced than the intratumoral variability of the parameters investigated, as indicated by large standard deviations of the mean regional concentration values. These respective data are summarized in table 1.

Table 1. Mean (±SD) concentrations of metabolites in 12 biopsies from cervical tumors of 10 patients as measured with quantitative bioluminescence and single-photon imaging in more viable or more necrotic tumor regions.

	ATP (μmole/g)	Glucose (μmole/g)	Lactate (μmole/g)
Viable	1.3 ± 0.6	3.0 ± 1.6	9.4 ± 4.4
Necrotic	0.3 ± 0.2	1.9 ± 0.8	3.3 ± 2.8

SD: standard deviation.

Most of the cases investigated showed a positive correlation between the distribution patterns of the three metabolites ATP, glucose and lactate; that is, there was a high concentration of ATP at a location with high glucose and lactate and vice versa. The quality of the correlation could be quantified by Spearman's correlation coefficient, r_s, which was 0.5 to 0.7 for the best correlations obtained. The average correlation coefficient form patients with clinically documented metastasis was 0.39. This was significantly higher than the average r_s value of 0.16 registered in patients without documented metastasis. Thus, better correlation between ATP and glucose seems to be an unfavorable prognostic parameter.

There were obvious differences in lactate distribution in tumors of patients with and without registered metastasis. As a representative example, figure 2 shows the frequency distribution in two cervical tumors generated from pixel values for lactate measured in these tumors. In patients without documented metastasis, relatively low lactate concentration values were measured within a relatively narrow concentration range. In contrast, very heterogeneous lactate distributions with values scattered over a wide range were obtained in patients with clinically documented metastasis. For the latter type of tumor, 13% of all values registered were in the range above 20 μmole/g, whereas only 2% of all measured lactate values were in this range in tumors without metastasis. Obviously, the presence of regions with very high lactate concentrations represents a risk of metastasis in cervical cancers.

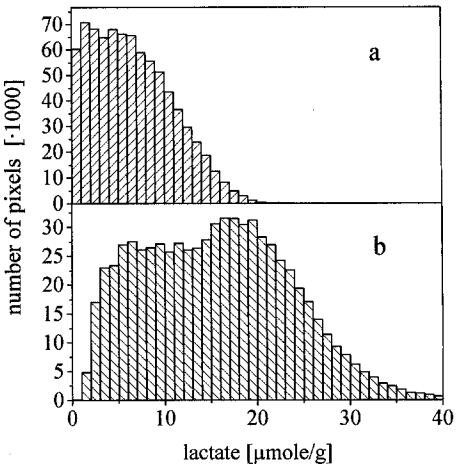

Figure 2. Frequency distributions of lactate concentrations in two cervical tumors of patients without clinically documented metastasis (*a*) and with metastasis (*b*).

Discussion

Quantitative bioluminescence and single-photon imaging offer the possibility of regional and structure-related evaluation of metabolite concentrations in biological tissue. The technique has been applied to tumors of rodent or human origin and various normal organs of laboratory animals. Distribution patterns of metabolites such as ATP, glucose and lactate in normal tissues were relatively homogeneous[11]. Regional variations in the concentrations of these metabolites were closely correlated with the histological zonation of the organs; for example, different concentration values were obtained in the cortex and in the medulla of the rat kidney. In contrast, metabolic patterns in malignant tumors showed pronounced heterogeneities. In many tumors, these patterns reflected the chaotic architecture of the malignancies[9]. Even in the rare tumors that exhibited a relatively homogeneous histological structure, distribution patterns of metabolites were extremely heterogeneous.

Previous investigations have demonstrated, at least for ATP, that this metabolite is positively correlated with tumor blood flow measured at a microregional level[15]. That is, the energetic status of malignant tissue is strongly influenced by the blood supply. In the present finding a statistically significant correlation between the glucose concentration in blood and that in tumor tissue was obtained. Such a correlation supports the finding that tumor blood supply is a major determinant of the metabolic milieu in tumors, which is obviously also true for tumors in patients.

The technique of quantitative bioluminescence and single-photon imaging has been designed for application to biopsies from tumors in the clinic. The present study demonstrates that this is feasible, and that metabolic imaging with this method can be performed adequately in human tumor biopsies. The results of metabolic imaging can be compared with data obtained from the clinical protocol, such as patient survival or incidence of metastasis. The present study on human cervical cancer showed no correlation between clinical staging or pathohistological grading of the tumors and any of the metabolic parameters measured. In general, local correlations between pixel values of ATP, glucose and lactate were positive, and these correlations were clearly better in patients with documented metastasis compared with patients without metastatic spread. The most striking difference between patients with and without metastasis was the difference in lactate distribution within tumor tissue. Whereas relatively low lactate concentra-

tions within a relatively narrow range of concentration were registered in malignancies of patients without metastasis, lactate concentrations obtained in patients with metastasis were scattered over a wide range of concentration. Frequency distributions obtained in patients without metastasis were tilted towards low lactate concentrations and rarely exceeded 20 µmole/g. In contrast, frequency distributions of lactate concentrations in tumors with metastatic spread were often extended between 0 and 40 µmole/g with a Gaussian shape or with two or more peaks. Obviously, high local levels of lactate within cervical cancers are associated with incidence of metastasis. This finding has still to be verified in a higher number of patients and also with different tumor entities. One hypothesis may be that increased levels of lactate, and reduced pH values, may decrease adhesive properties of tumor cells within malignant tissue and thus enhance the spread of cells from the primary tumor. One other possible explanation may be that unfavorable metabolic conditions within tumor tissue may enhance neovascularization, which may be true for both blood vessels and lymphatic vessels. Clinical investigations of tumor angiogenesis have demonstrated that increased neovascularization with regard to either blood or lymphatic vessels may be positively correlated with incidence of metastasis.

Metabolic imaging in human cervical cancer may therefore be indicative of the risk of metastatic spread. It may therefore help the oncologist to decide how aggressive therapy should be to fight the disease.

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