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Metabolism of peripheral lymphocytes, interleukin-2-activated lymphocytes and tumor-infiltrating lymphocytes from ^{31}P NMR studies

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^{31}P NMR spectra of tumor-infiltrating lymphocytes (TILs) were found to be significantly different from those of normal peripheral lymphocytes. The greatest difference was in the phosphodiester (PDE) region, mainly in the glycerophosphocholine (GPC) signal. Short-term activation of peripheral lymphocytes with interleukin-2 induced a small increase in ATP levels. In all lymphocytes the phosphomonoester (PME) region is dominated by phosphoethanolamine (PE), while there is an unusual absence of phosphocholine (PC). Perfusion of these cells with high concentrations of choline caused only a minimal increase in PC, indicating that choline kinase is not the rate limiting step of lecithin synthesis in lymphocytes.

Lymphocyte; Nuclear magnetic resonance, ^{31}P; Interleukin-2; Lymphocyte, tumor-infiltrating; Lymphocyte, IL-2 activated; Metabolism

1. INTRODUCTION

The development of adoptive immunotherapy has raised new hopes in the treatment of fatal diseases such as malignant melanoma [1]. The basis of this approach involves the activation of tumor-infiltrating lymphocytes (TILs) with a lymphokine, particularly interleukin-2 (IL-2). To date there is no information on the salient metabolic differences between normal, IL-2-activated lymphocytes (LAK), and TILs.

The ^{31}P NMR method is a valuable means to study the energy and phospholipid metabolism in intact viable cells, and can provide information on biochemical changes occurring during cell stimulation or inhibition [2]. The application of ^{31}P MRS in this study is an attempt to provide information on the metabolic status of the various kinds of lymphocytes, particularly their energy status (from the levels of ATP and P_i), and their phospholipid synthesis status (from their PME and PDE signals). Previously there have been a few attempts to study lymphocytes by these methods [3–5]. Such studies are not only of intrinsic interest, but may also provide information on the processes that are occurring in vivo, and might lead to non-invasive detection of changes using clinical in vivo MRS [5].

The purposes of this investigation were to define the ^{31}P MRS features of the various types of lymphocytes, to check whether there are significant MR spectral differences between them, to quantitate ATP levels of intact activated lymphocytes, and to investigate the enzymatic control of the phospholipid pathways in lymphocytes. The preliminary results obtained and presented here show that there are indeed significant differences in phospholipid metabolism between normal peripheral lymphocytes and TILs, as well as MRS detected changes in energy metabolism following lymphokine activation. To our knowledge these are the first reported ^{31}P MR spectra of TILs.

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2. MATERIALS AND METHODS

2.1. *Lymphocyte preparation*

Peripheral lymphocytes used in these studies were obtained from healthy volunteers through the NIH blood bank. The lymphocytes were collected through the apheresis technique, and separated and concentrated with the Ficoll-Paque method (Pharmacia LKB Biotechnology Inc.). For each NMR experiment $5 \pm 1 \times 10^8$ lymphocytes were used. For LAK cells, peripheral lymphocytes from patients with malignant melanoma were similarly isolated and cultured for 3–4 days in AIM-V growth medium (GIBCO Lab.) with 10^3 U/ml IL-2 (Human Recombinant Interleukin-2, Cetus Corporation) prior to the NMR measurements. The TILs which were studied here were from patients with metastatic malignant melanoma. Briefly, tumors were enzymatically dissected to single cell suspensions and placed into culture in AIM-V with 10^3 U/ml IL-2 and 20% LAK cell supernatant [6]. At one week cells were centrifuged and resuspended in fresh AIM-V with IL-2 and supernatant. When exponential growth of lymphocytes was observed at about 10 days TILs were maintained at densities between 5×10^5/ml and 2×10^6/ml by dilution into AIM-V with 10^3 U/ml IL-2. TILs were grown for 32–36 days prior to NMR studies. This was mostly due to the need to have a large number of lymphocytes for each experiment. Because of obvious clinical needs, the TILs used in these experiments were less than 5% of large TIL cultures used for patient treatments. Therefore, there were no NMR data of 'young' TILs.

2.2. *Preparations of lymphocytes for ^{31}P MRS*

Lymphocytes were harvested, centrifuged at 4°C at 1500 rpm for 10 min and washed 3 times in growth medium. Two methods were used:

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perfused lymphocytes and cell suspensions. Perfused lymphocytes were cast in agarose threads as follows [7]: cells were suspended in a low-gelling temperature agarose by mixing 0.6–0.8 ml of cells ($5 \pm 1 \times 10^8$) with 1.25 volumes of 3% agarose in phosphate-buffered saline at 37°C, and extruding the mixture through 0.5 mm internal diameter teflon tubing into a 10 mm NMR tube. The solid threads were concentrated without compression at the bottom of the NMR tube by using an insert with inlet and outlet tubing. The inflow tube (also 0.5 mm internal diameter) was placed near the bottom of the tube, and the outflow was directed into an opening in the insert. The perfusion solution consisted of Improved Minimal Eagle's Medium (IMEM), because of the need to know and control the concentrations of the constituents (the components of AIM-V have not been published). A peristaltic pump (Pharmacia P-3) maintained a constant perfusion rate of 0.5 ml/min. All experiments were performed at the physiologic temperature of 37°C. In each series of experiments control spectra were recorded prior to changing the perfusion solution. NMR studies of lymphocyte suspensions were done in order to investigate IL-2 effects, since IL-2 was considered to be too large (MW 18000) to penetrate the agarose threads. The lymphocytes were harvested and washed as described previously, and the pellet was transferred to a 10 mm NMR tube and diluted to a total volume of 2 ml with growth medium. Oxygen was continuously bubbled at the bottom of the tube to oxygenate the lymphocytes and to prevent settling. Each series of experiments with either perfused or suspended cells was repeated at least 3 times.

^{31}P spectra were recorded at 162 MHz on a Varian XL-400 NMR spectrometer. Each spectrum was collected for 400 or 800 scans (depending upon the amount of cells), using a 3 s repetition time, 35 ms pulse width (60°) and 20 Hz line broadening. ^{31}P chemical shifts were determined by standardizing glycerophosphocholine (GPC) to 0.49 ppm as an internal standard, and in some spectra where GPC was absent, by standardizing β-ATP to -18.7 ppm. For quantitative analysis peak integrals were measured, and MRS data acquisition and processing were performed with identical parameters throughout all experiments. After completion of the MRS the protein content of each sample was determined by the bicinchoninic acid assay (Pierce). This enabled standardization of the actual amount of lymphocytes used in each experiment, and made quantitative comparisons feasible.

3. RESULTS AND DISCUSSION

Lymphocytes are non-anchorage dependent cells and the preliminary set of experiments was designed to find the optimal conditions of casting to prevent loss of lymphocytes from the threads during perfusion. In our previous studies with human cancer cells [8], 1.8% agarose in equal volumes with the cells were used. However, because of the much smaller size of human lymphocytes, various agarose concentrations (1.8, 2.5, 3.0, 3.5%) and volumes were studied by NMR measurements concomitant with collecting and counting the lymphocytes that were washed out with the effluent. It was found that the optimal conditions were 3% agarose and 1.25:1 ratio of agarose to cell volume. However, even under the best conditions there was still a wash-out of ca. 1–2% of lymphocytes per hour, and therefore all experiments with threads were continued for only up to 12 h.

The ^{31}P MR spectrum of non-activated peripheral lymphocytes is shown in fig.1. The assignment of the signals was according to previous published data on lymphocytes [3]. The phosphomonoester (PME) region is governed by the phosphoethanolamine (PE) peak at

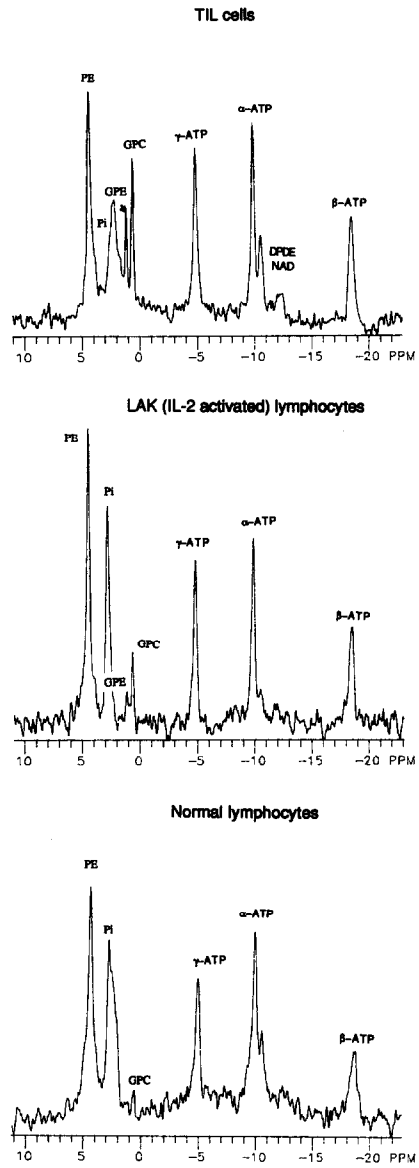

Fig.1. ^{31}P NMR spectra of normal lymphocytes, IL-2 activated lymphocytes and tumor-infiltrating lymphocytes (for acquisition parameters see section 2; LB = 20 Hz).

4.4 ± 0.1 ppm. Phosphocholine, a lecithin precursor, which is the dominant PME signal in most benign and malignant cells [8], is negligible in lymphocytes. Also typical to non-activated lymphocytes are the very low signals of the phosphodiester (PDE) degradation products of phospholipids, i.e. glycerophosphocholine (GPC) and glycerophosphoethanolamine (GPE). There is no phosphocreatine in lymphocytes, and we have obtained higher ATP levels compared to previously published data [3–5]. This finding of relatively high ATP concentrations, even in non-activated lymphocytes, was very reproducible. This is probably due to the large number of lymphocytes which were used in each experiment which gave a good signal-to-noise ratio, and to our improved method of lymphocyte

casting and perfusion. We wish to emphasize the importance of this point because another report tried to correlate ATP increase with IL-2 activation [5], but the initial low ATP levels may have resulted from methodological origins. It seems that a quantitative standardization method (for example, protein content determination) is essential for quantitative comparisons between different NMR measurements.

Lymphocytes cultured with IL-2 for 3–4 days exhibited some NMR spectral changes (fig.1), namely increased GPC and GPE signals. As described above, IL-2 effects on energy metabolism were investigated in lymphocyte suspensions. The energetic status was determined by the ATP/P_i ratio. Following the addition of IL-2 (10^5 units into the 2 ml in the tube) this ratio increased, but only by $20 \pm 12\%$ after 5 h (fig.2). Since these experiments were performed in cell suspensions they were conducted for relatively short duration (up to 5 h). Trypan blue staining at the end of the MRS studies showed that $90 \pm 5\%$ of the lymphocytes were viable. In LAK cells that were incubated with IL-2 for 3–5 days ATP/P_i ratios are in the range of 110–130% compared to non-activated lymphocytes. Thus it seems that IL-2 induces a small early increase in the levels of high energy compounds in lymphocytes. Under the experimental conditions of these studies only short term (hours) effects of IL-2 on lymphocytic energy metabolism could be investigated. Attempts are now underway to apply other techniques for prolonged (several days) lymphocyte perfusion in the NMR spectrometer.

TILs spectra were significantly different from spectra of peripheral and activated lymphocytes (fig.1), the main difference being a striking increase in GPC and GPE signals. These changes point to alterations in phospholipid metabolism, as GPC and GPE are the final phosphorous degradation products of phosphatidylcholine (lecithin) and phosphatidylethanolamine (cephalin), respectively [9]. These compounds are major constituents of membranes and the observed changes may reflect 'maturation' of the lymphocytes. However, it was previously postulated that GPC may also play a role in energy metabolism control, and act as a bioregulator of membranes [10,11]. Thus, investigating the remarkable increase of GPC in TILs may contribute to the understanding of the unique biological activity of these cells. In TILs as well as in the other lymphocyte types the PME region is governed by PE and there is almost no PC. There were no significant differences in ATP levels between TILs and LAK cells.

The metabolic pathways of phospholipids were studied in perfused TILs. In previous studies from our

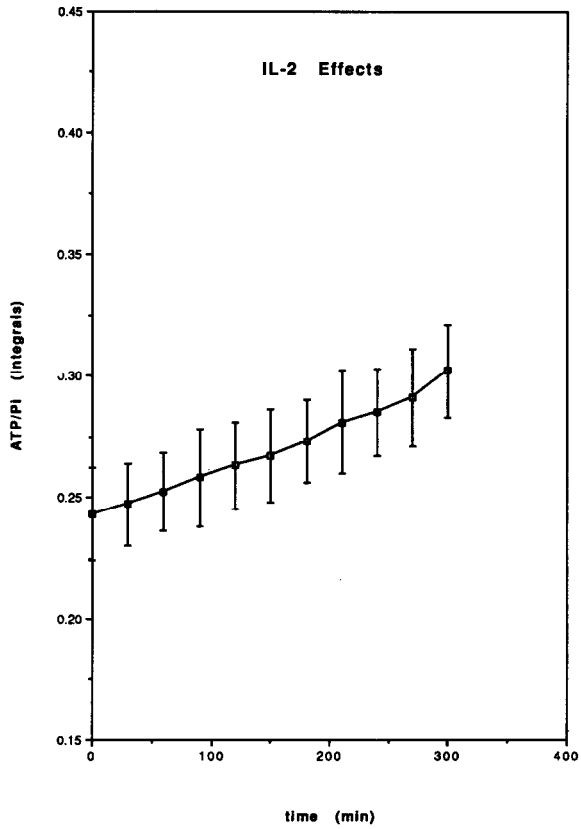

Fig.2. Changes in ratios of relative concentrations of ATP and inorganic phosphate following the addition of IL-2 into peripheral lymphocyte suspensions.

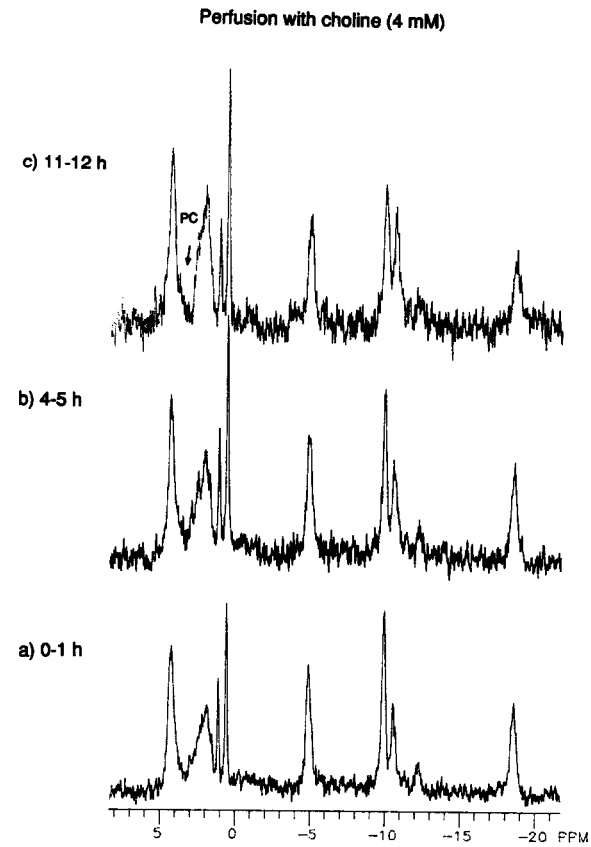

Fig.3. ^{31}P NMR spectra of TILs perfused with IMEM containing 4 mM choline (for acquisition parameters see section 2; LB = 5 Hz).

Table 1

The effects of 4 mM choline perfusion on the ratios of the NMR signals of TILs

	Control	After 12 h
PE/PC	2.87 ± 0.24	2.17 ± 0.21
PE/GPE	2.04 ± 0.19	1.43 ± 0.12
PC/GPC	0.37 ± 0.04	0.33 ± 0.05
GPC/GPE	1.84 ± 0.13	1.96 ± 0.14

laboratory, employing NMR techniques, it was found that in MDA-MB231 human breast cancer cells the rate-limiting enzyme of lecithin synthesis was cytidyltransferase, and the rate was modulated by PC levels [12]. In TILs PC concentrations are very low, even though there is choline in AIM-V (concentrations were not released), and the reason cannot be substrate depletion. We have perfused TILs with IMEM containing 4 mM choline (the physiologic concentration is 0.015 mM, and in normal IMEM there is 0.4 mM). This high concentration was used because choline is positively charged, and high intracellular levels were essential. Choline is an inhibitor of ethanolamine kinase and DPE diesterase [13]. Following perfusion with 4 mM choline-IMEM a small peak of PC appeared in the PME region (fig.3), but did not progressively increase throughout the experiments. Concomitant changes were PE decrease, and GPC and GPE increase as can be seen in table 1. These findings may indicate that the rate-limiting enzyme of lecithin synthesis in lymphocytes is different from that of other cells that were studied [9,12], since phosphocholine has not accumulated but rather is immediately converted into cytidyldiphosphocholine. This novel hypothesis is now being tested by using inhibitors of the terminal enzyme, DPE diesterase, of the lecithin synthetic pathway in NMR studies of perfused lymphocytes.

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