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Early Metabolic Response to Tumor Necrosis Factor in Mouse Sarcoma: A Phosphorus-31 Nuclear Magnetic Resonance Study[1]

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ABSTRACT

To investigate the effects of recombinant human tumor necrosis factor α (rHuTNF-α) on high-energy phosphate metabolism of cancer cells, ^{31}P nuclear magnetic resonance (NMR) studies were performed on a murine methylcholanthrene-induced sarcoma. Injection of 15 μg of rHuTNF-α caused progressive depletion of ATP and phosphocreatine within 90 min, together with an increase in inorganic phosphate. Metabolic changes were correlated with the early histological appearance of thrombosis and hemorrhage. A spatially localized NMR technique demonstrated that these changes were specific for the tumor. Acute ischemia of the tumor produced similar metabolic changes; thus the metabolic effects of rHuTNF-α could be due to either a primary action on tumor biochemistry or a secondary action produced by ischemia. These findings indicate that rHuTNF-α has a very rapid onset of action, which can be detected by ^{31}P NMR. Furthermore, the results suggest that ^{31}P NMR spectroscopy will be extremely useful for detecting early biochemical changes produced by rHuTNF-α or other treatments in animal and human cancers.

INTRODUCTION

The discoveries of a tumor necrosis-inducing substance in the sera of *Bacillus Calmette Guérin*- and endotoxin-treated mice, and of cytotoxic factors in cultures of activated lymphoid cells, resulted in the production of two related cytokines, rHuTNF-α[4] (also referred to as cachectin) and rHuTNF-β (also referred to as lymphotoxin) (1–3). These immune system hormones possess numerous biological activities (4–7). Apart from their direct cytotoxic actions against various tumor types, the *in vivo* antitumor activities of rHuTNF-α and rHuTNF-β may involve the induction of interleukin 1 production, activation of polymorphonuclear neutrophils, modulation of endothelial cell functions, and augmentation of specific immune functions (8–17).

Previous investigators using ^{31}P NMR have shown that chemotherapy, radiation, and hyperthermia may produce alterations of high-energy phosphate metabolism in experimental cancers (18–20). The results have been variable, depending on the type of cancer under study. Nevertheless, these findings suggest that metabolic measurements using ^{31}P NMR may provide insight into the mechanism by which chemotherapeutic agents kill neoplastic cells and may also indicate resistance or responsiveness of a cancer to a particular treatment program.

The goal of this study was to determine the extent and time course of metabolic alterations produced by rHuTNF-α in an animal Meth-A sarcoma. ^{31}P NMR was used to measure high-energy phosphates. The results show very early metabolic effects, suggesting a rapid onset of action for rHuTNF-α. The metabolic changes produced by rHuTNF-α are similar to those produced by ischemia, consistent with an effect on tumor blood supply.

MATERIALS AND METHODS

Animals. Experiments were performed on 6- to 8-wk-old female BALB/c × C57BL/6 F_1 mice, which were inoculated s.c. with 5×10^5 cells of a Meth-A sarcoma in the anterior body wall approximately 2 wk prior to NMR or histological experiments. At the time of ^{31}P NMR measurements, the tumors were 1 to 1.2 cm in diameter and discretely extended from the body wall. To study the effects of rHuTNF-α, the mice were restrained without anesthesia, and a 2-turn coil, 1.2 cm in diameter, was placed around the tumor. Three control spectra were initially obtained prior to rHuTNF-α injection. Following these measurements, mice were removed from the magnet, and 15 μg of rHuTNF-α (specific activity, determined by the L-M bioassay, was 5×10^7 units/mg; Genentech, Inc., South San Francisco, CA) were injected into a lateral tail vein. The mice were then returned to the magnet, and spectra were repeatedly obtained for approximately 5 h. For studies of tumor ischemia, mice were anesthetized using ketamine (2 mg) and rompun (0.2 mg) i.p. After control spectra were obtained, the mice were removed from the magnet and reanesthetized, and a suture was tightened around the base of the tumor to occlude the blood supply. Spectra were subsequently obtained in 21-min blocks.

NMR Methods. ^{31}P NMR spectra were obtained on a 5.6 Tesla 4-in horizontal bore magnet operating at 95.8 MHz. The acquisition pulse length was chosen to give a 90° pulse at the center of the coil. NMR spectra were acquired in 21-min blocks using 124 acquisitions, a 10-μs pulse, and a 10-s repetition time. For some studies a Depth Pulse A (21) was used to provide spectral data that were localized to the tumor. Where indicated, the rotating frame technique was used to obtain spectra from various (22) zones of the tumor and surrounding tissues. For all ^{31}P NMR data, the heights of various metabolite peaks were determined from the spectra after baseline flattening using convolution difference, except for experiments using the Depth Pulse A sequence, where the broad component was already eliminated by this pulse sequence.

RESULTS

The effects of rHuTNF-α on high-energy phosphates, measured by ^{31}P NMR, are illustrated in Fig. 1. To ensure that NMR signals were acquired primarily from tumor tissue (*i.e.*, to avoid signal contamination from adjacent nonmalignant tissue) a surface coil Depth Pulse sequence (21) was used. The control spectrum shows peaks for ATP, phosphocreatine, P_i, and phosphomonoesters. Following injection of rHuTNF-α, the spectrum obtained at 83 min shows an increase of P_i. This indicates a very early effect of rHuTNF-α on tumor energy metabolism. Subsequently, a continued increase in P_i was accompanied by a fall of both phosphocreatine and ATP. By 251 min virtually all ATP and phosphocreatine were depleted. The only remaining signals are a large P_i peak with a shoulder representing phosphomonoesters.

The time course of effects of rHuTNF-α on β-ATP, phosphocreatine, and P_i is shown in Fig. 2. By 2 h following injection

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[4] The abbreviations used are: rHuTNF-α, recombinant human tumor necrosis factor α; rHuTNF-β, recombinant human tumor necrosis factor β; Meth-A, methylcholanthrene induced.

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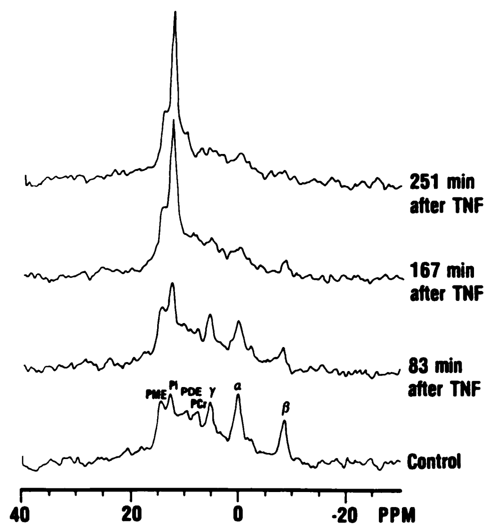

Fig. 1. Effects of rHuTNF-α on ^{31}P NMR spectra of Meth-A sarcoma. In the control spectra, α, β, and γ represent the three phosphates of ATP. *PDE*, phosphodiesters; *PME*, phosphomonoesters; *PCr*, phosphocreatine.

of rHuTNF-α, P_i had risen to 155 ± 11% of control, while phosphocreatine fell to 82 ± 4% and ATP fell to 65 ± 6% of control. These changes were observed in every tumor and indicate that rHuTNF-α produces profound alterations of high-energy phosphates. The long-term effects of rHuTNF-α on high-energy phosphorus metabolism were also examined in these tumors by a subsequent ^{31}P NMR measurement 17 to 20 h after injection of rHuTNF-α. These studies showed that phosphocreatine and ATP partially recovered, while P_i decreased (data not shown).

Histological examinations of s.c. implanted Meth-A sarcoma after rHuTNF-α treatment were performed at various time points (6). Table 1 shows that histological changes were detectable as early as 30 min after rHuTNF-α administration. The earliest changes were focal thrombosis and hemorrhage, followed by evidence of early focal necrosis. Fig. 3 shows histological changes 4 h after rHuTNF-α. Maximum necrosis and thrombosis were detected 24 h after rHuTNF-α treatment. Thrombosis and hemorrhage were seen only in tumors treated with rHuTNF-α. Thus, changes detected by light microscopy correlate well with the early changes detected by NMR, following rHuTNF-α administration.

Additional experiments were performed using multislice localization, termed "the rotating frame technique" (22), to separately monitor phosphate metabolites in the tumor and in adjacent nonmalignant tissue. Because the tumor was located in s.c. tissue, there was no appreciable NMR signal from tissue superficial to the tumor. Fig. 4*A* shows that ^{31}P NMR spectra of the cancer were separated from underlying muscle. Phosphocreatine was present in the tumor, but the phosphocreatine/ATP ratio was lower in the tumor than in adjacent normal muscle. Fig. 4*B* shows that, 4 h after rHuTNF-α administration, phosphocreatine and ATP were markedly reduced, while P_i increased throughout the entire tumor. In contrast, the spectra from adjacent muscle showed no significant changes in the relative intensities of phosphocreatine, ATP, or P_i. Furthermore, rHuTNF-α had no effect on the ^{31}P NMR spectrum of normal muscle, obtained with the surface coil on the opposite body wall (data not shown).

Acute ischemia produces rapid depletion of high-energy phosphates and a rise of P_i in a variety of tissues. To determine if the metabolic effects of rHuTNF-α resemble those of ischemia, ^{31}P NMR studies were performed in which the tumor blood

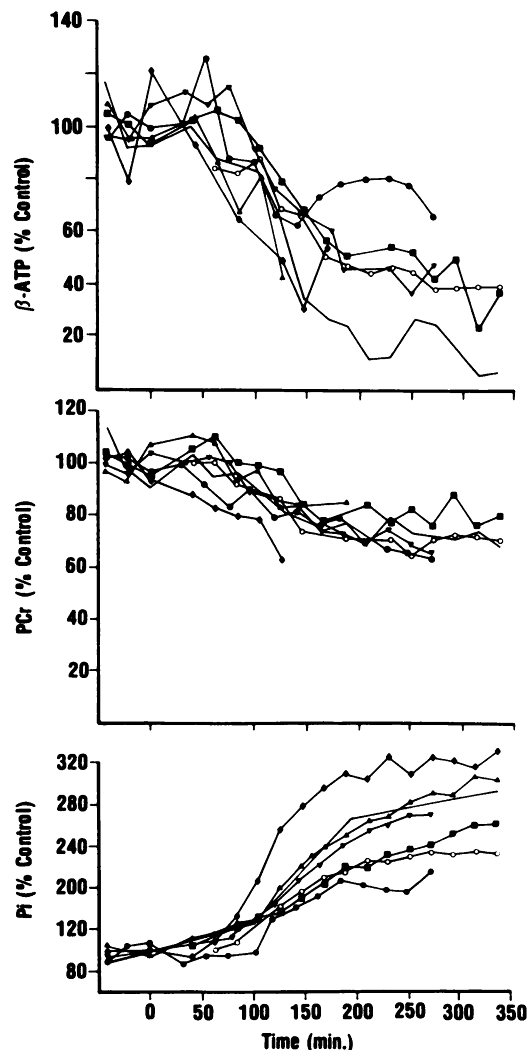

Fig. 2. Effects of rHuTNF-α on β-ATP, phosphocreatine (*PCr*), and P_i, expressed as a percentage of control, are depicted against time. The individual values for seven experiments are shown.

supply was acutely ligated. Fig. 5 shows that tumor ischemia caused rapid depletion of phosphocreatine and ATP accompanied by a rise of P_i in 1 h. The results were qualitatively similar to those produced by rHuTNF-α and suggest that the changes in high-energy phosphates induced in tumors are consistent with severe ischemia.

DISCUSSION

The important finding of these experiments is that rHuTNF-α produces rapid depletion of phosphocreatine and ATP and a

Table 1 *Semiquantitative grading of histological changes in s.c. implanted Meth-A sarcoma after rHuTNF-α administration*

Histological scoring was on a scale of 0 to 4+ with 1 representing mild and 4+ severe (1).

Time after rHuTNF-α treatment	i.v. treatment	Histological scoring		
		Necrosis	Thrombosis	Hemorrhage
15 min	rHuTNF-α	1.0	0	0
30 min	rHuTNF-α	1.0	1.0	0.3
60 min	rHuTNF-α	1.5	1.0	0.5
180 min	rHuTNF-α	2.4	2.7	2.4
360 min	rHuTNF-α	2.0	2.0	1.7
24 h	rHuTNF-α	3.4	3.0	1.0
	PBS[a]	1.0	0	0

[a] PBS, phosphate-buffered saline. Results are mean histological scores from phosphate-buffered saline-treated mice sampled at all time points indicated above.

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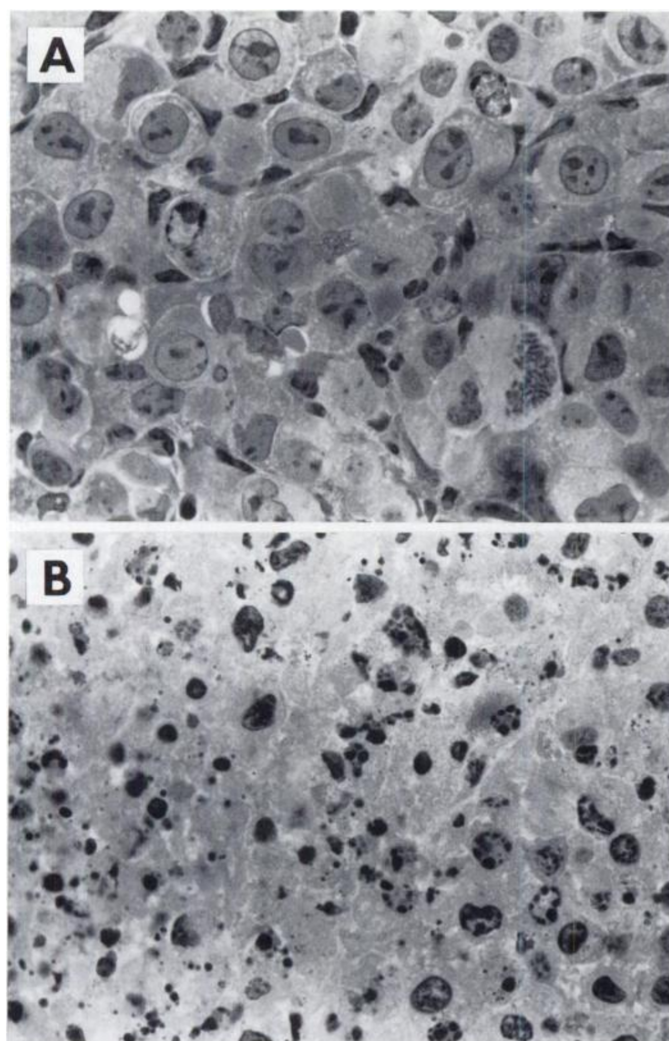

Fig. 3. Light microscopy of an actively growing nonnecrotic region (*A*) and a necrotic region (*B*) 4 h following rHuTNF-α administration × 6000.

rise of P_i, which are clearly observed by ^{31}P NMR 1 to 2 days before tumor shrinkage. The observed changes are similar to those produced by ischemic inhibition of energy metabolism. No changes of phosphocreatine, ATP, or P_i were observed in normal muscle, indicating a specific effect on malignant tissues. The results also suggest that ^{31}P NMR may be useful for the study of the mechanism of action of recombinant lymphokines and to provide an early indication of the responsiveness of tumors *in vivo*. These new observations must be considered in the general context of several mechanisms of action proposed for TNF.

A number of histological studies (23–25) have demonstrated hemorrhagic lesions in Meth-A tumor vasculature within 1 h after administration of TNF. Vascular damage would be expected to reduce the blood supply of the tumor, causing ischemia which would affect tumor energy metabolism. The results reported here are consistent with this hypothesis; changes in phosphocreatine, ATP, and P_i caused by TNF are qualitatively similar to those produced by ischemia. However, the present results are inconsistent with previous histological studies which indicate that the acute effects of TNF are confined to the core of the tumor (11, 24, 25) while the periphery is relatively unaffected. In contrast, the application of the rotating frame method reported here demonstrates that, after 3 h, TNF induced metabolic inhibition throughout the entire tumor. Partial metabolic recovery was observed at 24 h, even though histological evidence of thrombosis and necrosis was maximal at this point. This recovery may have occurred primarily at the periphery of the tumor, where vascular damage observed histologically is minimal (11, 24, 25) (the rotating frame experiment was not used to study tumors after 24 h).

Direct metabolic effects of TNF on tumor cells have also been reported. Beutler and Cerami (5) have equated TNF with cachectin and have suggested that TNF may be responsible for the cachexia produced by various diseases. Cachectin suppresses activity of lipoprotein lipase (26, 27) and may participate in mobilization of energy reserves by an infected host (5). Podo *et*

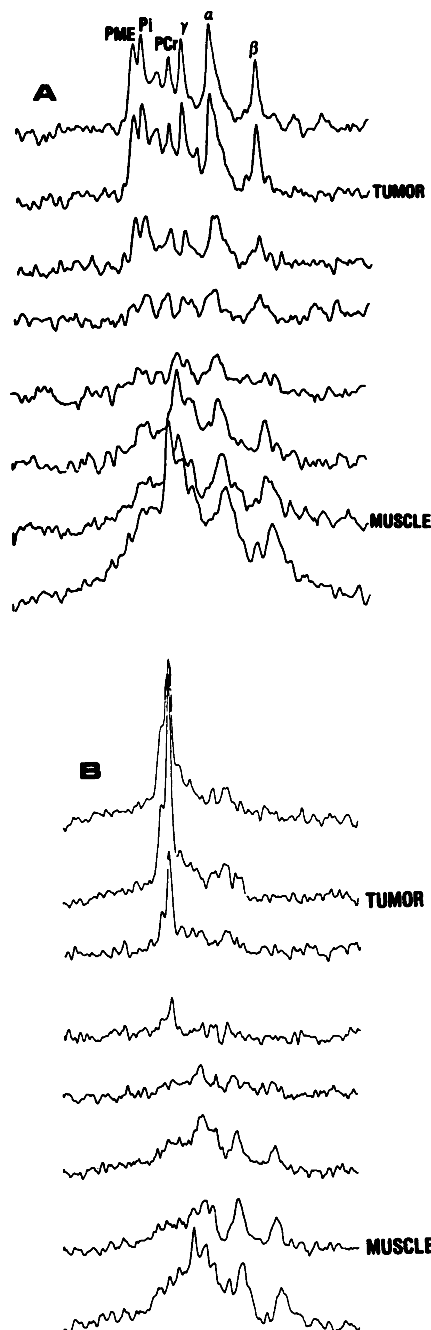

Fig. 4. Rotating frame experiment of Meth-A sarcoma *in vivo*. Spectra are depicted for the tumor and muscle tissue beneath the tumor. Peak assignments are similar to those in Fig. 1. Each spectrum represents a spatially localized slice from the top of the tumor, through the tumor, rib cage, and intercostal muscle (*bottom*). *A*, spatially resolved ^{31}P NMR spectra before rHuTNF-α; *B*, the corresponding spectra of the same tumor 4 h following injection of rHuTNF-α.

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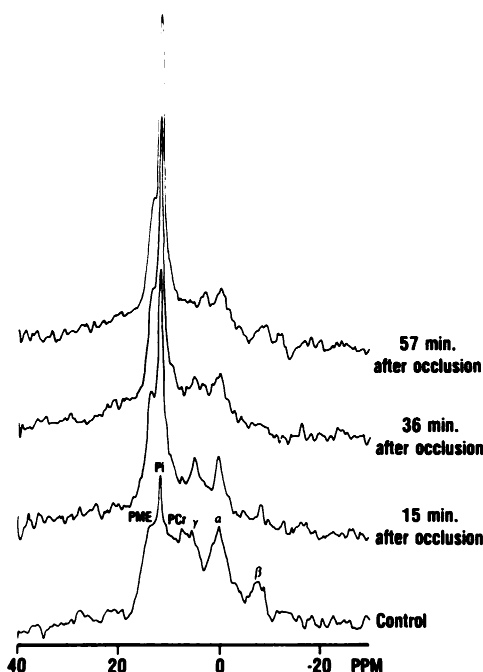

Fig. 5. Effects of ischemia on ^{31}P NMR spectra of Meth-A sarcoma.

al. (28) demonstrated that TNF alters phospholipid pool sizes in Friend leukemia cells. These changes in phospholipid biosynthesis and catabolism may also effect energy metabolism. The present results demonstrate acute metabolic effects of TNF *in vivo* and, thus, are consistent with the hypothesis that TNF directly affects energy metabolism.

Finally, it is possible that the action of rHuTNF-α is not directly related to intermediary metabolism. The changes in high-energy phosphate metabolism, as well as the hemorrhagic lesions observed histologically, may simply be secondary effects of tumor necrosis. Further experiments will be required to determine whether the acute effects of TNF on energy metabolism reported here reflect vascular damage and subsequent ischemia, direct metabolic effects of TNF, or secondary effects due to TNF-induced tumor necrosis.

The results of these experiments suggest that ^{31}P NMR may be applied to investigation of human tumors *in situ*. Presently the clinical response of tumors to therapy is assessed from patient survival and measurement of cancer size using scanning techniques. Following cancer treatment, several weeks to months are required to determine if there is a response to therapy. The observation that ^{31}P NMR detects early metabolic changes in responsive tumors (18–20) is strikingly confirmed by the current ^{31}P NMR measurements. The present results suggest that ^{31}P NMR of human tumors before, during, and following treatment may be used to identify tumors which do or do not respond. This may avoid unnecessary side effects and permit better design of treatment regimens for the individual patient based on the metabolic response of the tumor to therapy. Use of ^{31}P NMR to monitor cancer response during treatment may make it possible to individualize cancer treatment. Moreover, ^{31}P NMR may accelerate the rate at which different doses and combinations of drugs (including rHuTNF-α) or other therapies can be evaluated in clinical trials. Therefore, the application of NMR spectroscopy to cancer may provide new information on the mechanism of cancer treatment, improve the efficacy of patient therapy, and increase the efficiency with which new therapy for malignant tumors can be developed.

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