Uric Acid Promotes Tumor Immune Rejection

De-En Hu, Alistair M. Moore, Lindy L. Thomsen, and Kevin M. Brindle

1Department of Biochemistry, University of Cambridge, Cambridge, and 2Immunomodulation Section, Immunotherapeutics Department, GlaxoSmithKline, Stevenage, United Kingdom

Abstract

Uric acid released from dying cells has been shown recently to act as a danger signal for the immune system, stimulating dendritic cell maturation and enhancing T-cell responses to foreign antigens. Stimulation of dendritic cell maturation by uric acid has been proposed as a mechanism by which the immune system can generate responses against tumors. We show here that uric acid levels are elevated in tumors undergoing immune rejection and that the inhibition of uric acid production, by systemic administration of allopurinol, or the removal of uric acid, by administration of uricase, delayed tumor immune rejection, whereas subcutaneous administration of crystalline uric acid enhanced the rejection process.

Introduction

Coinjection of dying cells with an antigen into animals is known to have an adjuvant effect, stimulating T-cell responses to the antigen (1). Recently Shi et al. (2) purified this endogenous adjuvant activity from the cytosol of damaged cells and showed it to be uric acid, an end product of purine catabolism. This increases rapidly in concentration in injured cells as they degrade their RNA and DNA and explains previous observations that showed that the production of this endogenous adjuvant in injured cells did not require protein synthesis (1). Injection of purified uric acid was shown to boost CTL responses in splenocytes isolated from mice, which had been primed with particulate antigens, by triggering increased dendritic cell expression of the costimulatory molecules CD86 and CD80. Allopurinol and uricase treatment, which substantially reduced plasma uric acid concentrations, was shown to markedly inhibit this T-cell priming. The concentrations of uric acid that stimulated dendritic cells corresponded to the point at which uric acid crystals were precipitated, and it was shown that preformed crystals were highly stimulatory, whereas soluble uric acid was not. Uric acid crystals are known to stimulate monocytes to produce inflammatory mediators (3), and it seems likely that dendritic cells are stimulated in a similar way.

These data led Shi et al. (2) to propose a model for immune responses to tumors, in which dying tumor cells release uric acid and cellular antigens. The maturation of dendritic cells, which acquire these antigens and present them to the immune system, is promoted by uric acid; thus, stimulating the immune response to the tumor. We have investigated this model here by determining whether uric acid levels are increased in a tumor undergoing immune rejection and by examining the effects of increasing or decreasing uric acid levels on the rejection process.

Materials and Methods

Cell Lines and Tumor Implantation. The E.G7-OVA cell line was derived from the murine thymoma line EL-4 by transfection with a neomycin-selectable vector expressing full-length chicken ovalbumin (4). Cells were cultured as a suspension in RPMI 1640 (In vitrogen Ltd., Paisley, United Kingdom) containing 10% heat-inactivated fetal calf serum (PAA Laboratories Ltd., Yeovil, Somerset, United Kingdom), 2 mM l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. Clones expressing different levels of surface ovalbumin were selected as described previously (5). Wild-type female C57BL/6 mice were purchased at 6–8 weeks of age from Charles River Ltd. (Thanet, United Kingdom) and tumor cells (5 × 10⁶) were injected subcutaneously into their shaved flanks. Tumor size is reported as the product of the two largest perpendicular diameters (in square millimeters). Rasburicase, 1.5 mg/ml, a recombinant form of urate oxidase, was obtained from Sanofi-Synthelabo Inc. (New York, NY).

All of the experiments were conducted in compliance with a project license issued under the Animals (Scientific Procedures) Act 1986 and were designed with reference to the UK Coordinating Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia. The work was approved by a local ethical review committee.

Tumor Histology. Tumors were fixed in 10% formalin and embedded in paraffin. Five-micrometer-thick sections were cut and stained with H&E or Masson’s trichrome stain.

Induction and Measurement of Apoptosis in Vitro and in Vivo. Cultured cells were induced to undergo apoptosis at a density of 10⁶ cells/ml by the addition of 25 µM etoposide to the culture medium. To induce tumor apoptosis, we injected etoposide, 67 mg/kg, and cyclophosphamide, 100 mg/kg, into mice 24 hours prior to tumor excision. Cell apoptosis was scored by monitoring nuclear fragmentation, after cell staining with 50 µg/ml propidium iodide and 10 µg/ml acidine orange. Cells that possessed condensed or fragmented nuclei but intact plasma membranes were scored as apoptotic (6). Tumor apoptosis was scored by counting the number of cells with condensed and fragmented nuclei in sections stained with Masson’s trichrome.

Measurement of Uric Acid Concentrations in Cell and Tumor Extracts. Tumor tissue was mechanically homogenized [1 g of tissue in 2.5 ml of ice-cold buffer containing 250 mM Tris-HCl (pH 7.4), 10 mM EDTA, 10 mM EGTA, and 1 ml/50 ml protease inhibitor cocktail (Sigma)]; the homogenate was centrifuged at 16,000 × g, and the supernatants were passed through 0.45 µm Millex filters (Millipore Corporation, Bedford, MA). Cultured cells were harvested and washed in ice-cold PBS, and then ~2 × 10⁶ cells were resuspended in 2 ml of fresh extraction buffer. The buffer contained 50 mM Tris-HCl (pH 8.2), 2 mM DTT, 2 mM EDTA, and 1% Triton X-100. After a 10-second homogenization, the resulting extracts were kept on ice for 30 min and then were centrifuged for 15 minutes at 2,000 × g. The supernatants from cell and tumor homogenates were removed and were assayed for uric acid enzymatically (assay kit from ThermoTrace, Melbourne, Victoria, Australia).

Preparation of Uric Acid and Monosodium Urate Crystals. Monosodium urate crystals were prepared by dissolving uric acid at a concentration of 5 mg/ml in 0.1 M sodium borate buffer, pH 8.5–9. The solution was then warmed to 55°C and, after filtering, the supernatant was left to sit for more than 72 h, whereupon monosodium urate crystals formed.

Cell Growth Rates in Vitro. These were determined either by cell counting under the microscope or by use of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MTT was added to cell suspensions at a final concentration of 0.5 mg/ml, and the mixture was incubated for 4 hours at 37°C. The cells were then harvested by centrifugation, the pellet was resuspended in DMSO, and the absorbance was measured at 540 nm.
Results

Induction of Tumor Apoptosis Results in Uric Acid Production. Etoposide-induced apoptosis in EL-4 cells resulted in significant accumulation of uric acid, when compared with control cells (Fig. 1A; n = 9, P < 0.01). The amounts were comparable with those observed previously in damaged EL-4 cells (2). Cells were harvested 18 h after etoposide addition, when fluorescence microscopy measurements indicated that 41 ± 2% of the cells were apoptotic and 19 ± 2% necrotic. Treatment of implanted EL-4 tumors with etoposide and cyclophosphamide also resulted in significant uric acid accumulation (n = 8), when compared with untreated tumors (n = 7, P < 0.01; Fig. 1B). Histological analysis of tumor sections indicated that ~38 ± 1% (30 sections from 3 tumors) of the cells showed signs of apoptosis (cell shrinkage and nuclear condensation).

Uric Acid Concentration Increases in Tumors Undergoing Immune Rejection. EL-4 tumor cells can be rendered immunogenic by transfection with a vector expressing chicken ovalbumin (E.G7-OVA; 4). We showed previously that by cloning cells with different levels of surface ovalbumin expression we were able to titrate the immunogenicity of this tumor to produce a range of subcutaneous tumors that showed different frequencies of immune rejection (5, 7). Two lines were selected for the experiments described here. A line at nominal passage number 14, that produced tumors of which were selected for the experiments described here. A line at nominal treatment with 25 μ g/kg etoposide and 100 μg/kg cyclophosphamide into mice 13–15 days after tumor implantation and 24 h before tumor excision. Uric acid concentrations were determined enzymatically in tumor and cell homogenates (cells, n = 9; control tumors, n = 7; apoptotic tumors, n = 8).

Statistical Analysis. Statistical analyses were performed using GraphPad Prism Software (Graphpad Software, Inc., San Diego, CA). ANOVA and t test were used to compare specific groups. Values shown are the means ± SE.

Discussion

According to the “danger model,” antigen-presenting cells are activated by danger or alarm signals released from injured cells to
produce costimulatory molecules that promote T-cell responses to a copresented antigen (8, 9). Recently Shi et al. (2) identified uric acid as the principal endogenous danger signal released from injured cells. They showed that it stimulated dendritic cell maturation in vitro, increasing the expression of the costimulatory molecules, CD86 and CD80, and, when coinjected with a particulate antigen into mice, it enhanced the generation of a CD8+ T-cell response. Furthermore, the priming of an antigen-specific T-cell response, by coinjection of injured cells, was substantially reduced by treatment of the animals with allopurinol and uricase, which markedly decreased the plasma concentrations of uric acid. They suggested a “danger signal” model in which the release of uric acid by injured cells, produced as a result of DNA and RNA degradation, stimulates the maturation of dendritic cells, which also acquire and present antigens from the dying cells to T cells, thus enhancing the immune response. If, in the case of a...
tumor, the host is not tolerant of these antigens, then an immune response against the tumor will be generated.

We have shown here that tumors in which there is increased cell death, either as a result of treatment with a chemotherapeutic drug or as a consequence of immune rejection, show elevated levels of uric acid. This increase in uric acid production seemed to be involved in tumor immune rejection because treatment of mice bearing a highly immunogenic tumor, with allopurinol or uricase [which have been shown previously to lower plasma uric acid levels (2)] significantly delayed tumor regression. Although allopurinol may have immunomodulatory effects that are unrelated to its effect on systemic levels of uric acid (10), this is unlikely to be the case for uricase. Furthermore, Shi et al. (2) showed that the CTL response in splenocytes isolated from mice, immunized with activated dendritic cells that had been pulsed with a peptide corresponding to the antigenic epitope, was undiminished by treatment of the animals with allopurinol plus uricase. In further support of a role for uric acid in the immune rejection of this tumor, we have shown that subcutaneous inoculation of uric acid crystals, 2 cm from the site of tumor implantation, increases significantly the frequency of tumor immune rejection.

There is, however, a paradox in the role of uric acid as an enhancer of tumor immune rejection. We have shown previously that immune rejection of the E.G7-OVA tumor model involves the production of nitric oxide by the tumor cells. This is involved in their death, probably as a result of combination with superoxide to form peroxynitrite (5), which is a potent cellular oxidant and mediator of cell death (11). Uric acid is a natural peroxynitrite scavenger (12) and, therefore, might have been expected to protect the cells from immune cell-mediated death. Such a role is inconsistent with the observations that uricase and allopurinol treatment, which lowers plasma uric acid levels, delayed tumor immune rejection and that inoculation of uric acid crystals promoted rejection. Shi et al. (2) suggested that uric acid stimulated dendritic cell maturation only when crystalline and proposed that this chemical phase transition could be the key event that turns uric acid into a danger signal. Therefore, perhaps the role of uric acid as an antioxidant or as a stimulator of the immune response depends crucially on this transition, which will in turn depend on other factors in the tumor microenvironment and the surrounding tissue (2).

References