Basic Fibroblast Growth Factor Secreted by an Animal Tumor Is Detectable in Urine

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ABSTRACT

Basic fibroblast growth factor (bFGF or FGF-2) is abnormally elevated in the serum and urine of patients with many types of cancer. However, the source of the bFGF is unclear. We developed a model that could distinguish between tumor-derived and host-derived bFGF. We gave athymic mice s.c. injections of cells of the murine K1000 tumor, which secretes a bFGF mutein (bFGF CS23) as its dominant angiogenic factor. Controls were given injections of Lewis lung carcinoma or saline. Urine was collected for 9 weeks, and bFGF was quantitated using two immunoassays which can discriminate between tumor bFGF CS23 and native bFGF. None of the mice had detectable urinary native bFGF, and no control mice had detectable urinary bFGF CS23. In contrast, urine from mice bearing the K1000 tumor revealed detectable bFGF CS23 by 2 weeks, and bFGF CS23 increased with increasing tumor volume throughout the study. Because bFGF CS23 is not produced by other cells, the bFGF CS23 in the urine most likely came from the K1000 tumor and not from the host. These results suggest that the source of elevated bFGF in the urine of human cancer patients is, at least in part, the tumor itself.

INTRODUCTION

A wealth of clinical and experimental evidence is accruing that tumor growth is dependent upon the process of angiogenesis (1-3) and that this process is mediated by diffusible factors (4). At least eight angiogenic peptides have been sequenced and cloned, and several low molecular weight nonpeptide angiogenic molecules have also been identified (5). Until recently, however, there has been little direct evidence to indicate which of these angiogenic factors, if any, are utilized by tumors to induce neovascularization in vivo.

A compelling demonstration that basic fibroblast growth factor can mediate angiogenesis was recently reported. Leader-sequence-fused bFGF3 complementary DNA was transfected into murine fibroblasts (6), transforming them into K1000 cells which use the new signal peptide to secrete a serum-substituted bFGF mutein (bFGF CS23) as their dominant angiogenic factor. Angiogenic activity and growth of the K1000 tumor were significantly inhibited by treatment of the mouse with a neutralizing antibody specific to bFGF CS23 (7).

There is also new evidence to suggest that bFGF is secreted by human tumors. Chodak et al. (8) demonstrated elevated bFGF-like activity in the urine of patients with bladder or kidney cancer. Using a sensitive bFGF sandwich EIA (9), Fujimoto et al. (10) reported high levels of bFGF in the serum of patients with renal cell carcinoma. We used the same assay in a wider spectrum of cancers and found bFGF to be abnormally high in the serum (11) and urine (12, 13) of patients with many types of cancer. These findings do not, however, reveal the source of the bFGF. The bFGF detected in the serum and urine of cancer patients could be coming from: (a) tumor cells; (b) vascular endothelial or other host cells recruited to the tumor; (c) both tumor cells and host cells; or (d) some other source, such as the extracellular matrix.

To address this question, we attempted to develop an animal model of urinary excretion of bFGF which could distinguish between tumor-derived bFGF and host-derived bFGF. We took advantage of the K1000 animal-tumor model (7) and two sandwich EIAs which can quantitatively discriminate between the bFGF CS234 secreted by K1000 cells and native bFGF (9) synthesized by the host, to test the hypothesis that tumor-derived bFGF can be detected in the urine of tumor-bearing mice.

MATERIALS AND METHODS

Materials. Recombinant human bFGF (14) and its bFGF CS23 mutein, in which serine is substituted for cysteine at residues 70 and 88 (15), have equivalent biological activity (15). The preparation of the sandwich EIA for recombinant human bFGF was prepared as described (9); the sandwich EIA for bFGF CS23 was prepared in a similar fashion.4 Both are sensitive to 100 pg/liter.

Tumor Lines. The murine 3T3 fibroblast cell line, A31, was transfected with the pTB1000 expression vector to produce the K1000 cell line (6). K1000 cells use the signal sequence for human interleukin 2 to secrete bFGF CS23 via the classical secretory pathway (7). The murine Lewis lung carcinoma cell line was purchased from the American Type Culture Collection. Both cell lines were maintained in Dulbecco's modified Eagle's medium with 10% heat-inactivated fetal calf serum.

Urine Collection. Six-week-old male athymic BALB/c-nu/nu mice were purchased from the animal breeding facilities at Massachusetts General Hospital (Boston, MA) and were treated in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals. The mice were fed rodent chow ad libitum and had continuous access to 10% sucrose solution to increase spontaneous drinking and, consequently, urine output. Two groups of four mice received 0.1 ml dorsal s.c. injections of 3 × 106 cultured cells in PBS; one group was given an injection of K1000, the other of Lewis lung carcinoma. Four control animals were given injections of PBS alone. Tumor measurements were made with calipers, and tumor volume was calculated using the formula $v = \frac{w^2l}{2}$. The mice were maintained in stainless steel metabolic cages from which stool-free urine drained continually into glass collection tubes. Animals were sacrificed by cervical dislocation at 9 weeks.

Urine Processing. Urine was collected daily and frozen at −20°C until the conclusion of the trial. The weekly urine from each animal was combined for testing. One ml of each sample was analyzed for creatinine concentration by an Automated Clinical Analyzer (Dupont, Wilmington, DE). The remaining urine was centrifuged at 3000 rpm for 8 min at 4°C, and the sediment was discarded. The supernatant was passed through a 1.2 μm low protein-binding filter (Gelman Sciences, Ann Arbor, MI), dialyzed using Spectra/por (Spectrum Medical Industries, Los Angeles, CA) cellophane dialysis tubing (molecular weight cutoff, 6000–8000) for 24 h at 4°C against 3 changes of distilled water, and lyophilized to dryness. The lyophilized was adjusted to 1% of the original volume with a solution of 25% Block Ace (bovine milk protein; Snow Brand, Inc., Japan), 100 μg/ml heparin, and 10 μg/ml mouse IgG in PBS. Samples were assayed using the sandwich EIAs for bFGF CS23 and bFGF, and mutein forms of basic fibroblast growth factor, manuscript in preparation.
recombinant human bFGF, and the resulting values were normalized against urine creatinine using the formula.

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\text{Normalized bFGF (pg/g)} = \frac{\text{pg of bFGF/liter of urine}}{\text{g of creatinine/liter of urine}}
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RESULTS

Tumor Growth and Metastases. Subcutaneous tumors were detected in both K1000 and Lewis lung carcinoma animals after 5–7 days. At sacrifice, mice bearing K1000 tumors had 0–2 surface lung metastases, while mice bearing Lewis lung carcinoma had 3–6 surface lung or liver metastases. No animals had any evidence of metastases to kidney, ureter, or bladder.

Detection of Urine bFGF. The daily production 5–8 ml of urine/mouse/day was not affected by tumor volume. It should be noted that normalization for creatinine, which corrects for the hydration status of the mouse, artificially increases the baseline level of bFGF (Fig. 1). No animals had any detectable native bFGF, and neither Lewis lung carcinoma nor control animals had any detectable bFGF CS23 in their urine at any point during the study. In contrast, the mice bearing K1000 tumors had detectable bFGF CS23 in their urine as early as the second week after tumor inoculation (Fig. 1), and their bFGF CS23 levels continued to increase with increasing tumor volume throughout the study (Fig. 2). Maximum urine bFGF CS23 levels of three of these mice were approximately 60, 300, and 9800 times higher than the baseline.

DISCUSSION

We have detected high levels of bFGF CS23 in the urine of animals bearing tumors bioengineered to secrete a mutagenized but biologically active (15) form of bFGF. Prior to this report, there was no method to label a circulating angiogenic factor secreted by a tumor. The specific immunoassays for the mutein and native forms effectively distinguish between tumor-derived and host-derived bFGF. Because the mutein bFGF CS23 is not produced naturally by any other cells, the bFGF CS23 detected in the urine of the study mice most likely came from the K1000 tumor per se and not from the host endothelial cells or from some other source.

Native bFGF is found predominantly within the cell (16–18) and occasionally in the basement membrane (19) and extracellular matrix (20). The fact that it is not usually secreted by normal cells is presumed to be due to its lack of a signal peptide necessary for the classical endoplasmic reticulum-Golgi pathway (21, 22). The export of bFGF by transfection of a secretory signal sequence along with complementary DNA for native bFGF (23) or for a bFGF mutein (6, 7) yields cells that are highly tumorigenic. By combining the injection of K1000 cells into nude mice with an assay specific to bFGF CS23, we have created an animal model of urinary excretion of tumor-derived bFGF. Because this model has certain similarities to cancer patients with elevated urinary bFGF (8, 12, 13), it provides indirect evidence that the elevated bFGF detected in the urine of human cancer patients may be arising, at least in part, from the tumor itself.

Of interest was the wide variability in the production of bFGF by K1000 tumors of similar volume. One mouse bearing a K1000 tumor excreted urine with bFGF CS23 levels 30–160 times higher than urine from other mice bearing equivalent-sized K1000 tumors, with a comparable degree of vascularity and number of metastases. Despite the small sample size, this disparity suggests that there is heterogeneity of angiogenic activity (3). This could be due to differential secretion from viable cells5 or to variable release from differential necrosis within each tumor. Alternatively, configurational changes in the secreted bFGF might render it undetectable to our assays.

Equally interesting was the fact that another aggressive tumor, Lewis lung carcinoma, produced no detectable urinary bFGF at all. Basic FGF cannot be detected in the conditioned medium or cell lysate of cultured Lewis lung carcinoma cells. Since this tumor is highly vascularized and grows rapidly, Lewis lung carcinoma must use a molecule other than bFGF as its angiogenic factor.

We were also intrigued by the apparent stability of bFGF in urine, since its half-life in distilled water6 and in the bloodstream (24) is measured in minutes. Heparin is known to protect bFGF against degradation (25); therefore one possible explanation is that heparin-
related compounds found in urine (26) are able to stabilize bFGF, but this remains to be demonstrated.

This model holds promise as a relatively simple technique for measuring the amount of bFGF produced by a tumor. Urine is easier to collect than serum, and it allows for serial bFGF determinations, even in a small animal like the mouse. In addition, the stability of bFGF in urine permits infrequent collection. The quantitation of urinary levels of bFGF should prove useful as a measure of the efficacy of experimental therapy; if it does, it may well find utility as a prognostic marker to guide the care of cancer patients.

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