A Synthetic Matrix Metalloproteinase Inhibitor Decreases Tumor Burden and Prolongs Survival of Mice Bearing Human Ovarian Carcinoma Xenografts

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ABSTRACT

We have examined the effect of a synthetic low-molecular-weight matrix metalloproteinase inhibitor, [4-(N-hydroxyamino)-2R-isobutyl-3S-(thiopen-2-ylthiomethyl)-succinyl]-l-phenylalanine-N-methylamide (BB-94), on human ovarian carcinoma xenografts growing in nude mice. The xenografts grew as thick intraperitoneal mucinous ascites containing free-floating tumor cell clumps. The ascites increased in volume, causing death approximately 3 weeks after introduction. Treatment with BB-94 caused resolution of ascitic disease. Tumor burden was dramatically reduced, and survival increased 5-6-fold. The increase in survival was dose dependent. The effects observed with BB-94 appeared to be due to its matrix metalloproteinase inhibiting effects, inasmuch as its inactive diastereoisomer had no effect on tumor biology. Following treatment with BB-94, free-floating clumps of tumor cells became surrounded by a capsule of host cells. These clumps of tumor cells typically formed one small (approximately 8 mm) avascular tumor of bright white appearance loosely attached to the peritoneum. Tumor cells within these capsules often appeared to be necrotic. Gel substrate analysis demonstrated that activated M, 92,000 type IV collagenase was present in the xenografts. We propose that inhibition of this enzyme causes the transition of ascites to solid tumors, concomitantly slowing tumor cell growth and allowing the development of tumor stroma.

INTRODUCTION

MMPs are a family of homologous enzymes capable of degrading components of the extracellular matrix. Different MMPs share highly homologous zinc-binding active sites, hemepxin-like domains, and cleavable NH_2-terminal sequences the removal of which results in activation of the enzymes. Members of the MMP family are distinguished by their substrate specificities. Intestinal collagenase (MMP-1) degrades interstitial collagen and is the only MMP capable of doing so. Both M, 72,000 and M, 92,000 type IV collagenases (MMP-2 and MMP-9, respectively) digest types IV and V collagen and to a lesser extent proteoglycans and fibronectin (Refs. 1 and 2 and references therein), while stromelysin (MMP-3) preferentially degrades proteoglycan and fibronectin and has limited degradative effects on type IV collagen (3).

MMPs are involved in tissue remodeling during wound healing (4) and embryogenesis (5). They also contribute to the damage which occurs in rheumatoid arthritis (6) and facilitate the passage of tumor cells across the basement membrane in metastasis (7-9).

Given the involvement of MMPs in several disease states, considerable interest has developed in the therapeutic potential of synthetic inhibitors. Inhibition of MMPs would be expected to limit the formation of metastasis by preventing basement membrane degradation and may have additional beneficial effects by inhibiting angiogenesis (10) and promoting the formation of stroma causing encapsulation of the tumor. BB-94 is a low-molecular-weight (MW = 478) synthetic compound which inhibits a broad spectrum of MMPs. Here we report that this compound radically changes the composition of stroma and the biological behavior of ovarian carcinoma xenografts grown in nude mice, resulting in a dramatic reduction in tumor burden and a concomitant increase in survival.

MATERIALS AND METHODS

Ovarian Carcinoma Xenografts. The xenografts were established from human primary ovarian tumors. Both were maintained and passed i.p. LA was established from a 72-year-old woman with a poorly differentiated mucinous cystadenocarcinoma, and HU was established from a 23-year-old woman with a moderately differentiated serous cystadenocarcinoma (11).

BB-94. BB-94 was provided by British Biotechnology, Ltd. (Cowley, Oxford, England). The structural formula of this compound is given in Fig. 1. Essentially, BB-94 contains a peptide backbone which binds the molecule to matrix metalloproteinases and a hydroxamic acid group which binds to the catalytically active zinc atom. The concentrations (ms) of BB-94 producing 50% inhibition against the following matrix metalloproteinases are: interstitial collagenase, 3; stromelysin, 20; M, 72,000 type IV collagenase, 4; M, 92,000 type IV collagenase, 1-10. BB-94 is a fine white powder and was sonicated into suspension at 2.5 mg/ml in phosphate-buffered saline (pH 7.2) plus Tween-20 (0.01%). This preparation was injected into nude mice i.p. An inactive diastereoisomer of BB-94, BB-1268 was prepared in an identical way.

Gel Substrate Analysis. Gel substrate analysis was performed essentially as described by Brown et al. (12). Solid tumor was removed from BB-94-treated HU-bearing mice and homogenized in SDS electrophoresis sample buffer (0.15 m Tris, pH 8.8; 10% glycerol, v/v; 1% SDS w/v). Homogenized samples were applied directly without heating or reduction to a 5% stacking polyacrylamide gel laid over an 11% (w/v) polyacrylamide gel containing 1 mg/ml gelatin and 0.1% (w/v) SDS. Gels were run at room temperature at 180 V. After incubation of gels in 2.5% Triton X-100 for 30 min to remove SDS, the gels were incubated for 16 h in 50 ms Tris-HCl (pH 7.6) containing 0.2 m NaCl, 5 ms CaCl_2, and 0.02% (w/v) Brj-35. Gels were stained for 3 h in 30% methanol/10% glacial acetic acid containing 0.5% (w/v) Coomassie brilliant blue G 250 and destained in the same solution in the absence of dye.

RESULTS

Effect of BB-94 on Biology of Xenografts. The HU and LA xenografts grew as thick mucinous ascites in the peritoneal cavity of the nude mouse. Seven days after introduction of the HU xenograft, the peritoneal cavity of the host contained 1-2 ml of mucinous ascites. By 14 days this had increased to 3-4 ml and by 21 days to 5-6 ml. Treatment of mice with BB-94 i.p. daily from 3 days after tumor transplantation prevented formation of ascites. A small volume of solid tumor of bright white appearance was visible in these mice, and the burden of tumor was not appreciably greater on day 21 than on day 7. Fourteen days after introduction of the HU xenograft, the volume of accumulated ascites in the untreated animals was sufficient to cause the abdomen to become visibly swollen. However, when the animals were treated with BB-94, their abdomens did not swell and they appeared healthy. Fig. 2 shows the appearance of the peritoneal cavity.
of mice bearing the HU xenograft at 14 days after treatment with vehicle alone (Fig. 2a) or with BB-94 (Fig. 2b). The large arrow indicates the position of solid tumor. In the animals treated with vehicle alone, mucinous ascites covered all of the peritoneal organs, while the tumor burden of BB-94-treated animals was low; typically each animal had one large tumor of maximum dimension ~8 mm which was free floating in the peritoneal cavity or attached to fat. Such tumors were located at the rectum end of the colon or at the base of the spleen. They were only loosely attached to the fat and were avascular, which caused them to appear bright white. A similar pattern was observed with the LA xenograft. Mice treated with diluent alone became swollen by 14 days due to an accumulation of mucinous ascites, whereas those treated with BB-94 appeared normal. Post mortem revealed solid white tumors loosely attached to peritoneal organs, typically at the colon and base of the spleen. Small white spots (<1 mm maximum dimension) were visible in all BB-94-treated mice (Fig. 2b, small arrows). It was confirmed histologically that these were not tumors or inflammatory infiltrates but accumulations of drug. These were also observed in non-tumor-bearing mice treated with BB-94.

Following i.p. injection of BB-94, an influx of PMN into the peritoneal cavity was observed. Twenty-four h after i.p. administration of BB-94, the percentage of peritoneal cells which were PMN changed from 6.6 (vehicle only) to 53.2. This was the only significant change in peritoneal cell population induced by the drug.

Effect of BB-94 on Tumor Cells in Vitro. The observed antitumor effects of BB-94 were unlikely to be caused by any direct cytotoxic effect toward the tumor cells themselves. BB-94 had no effect on the growth of various cell lines tested including SKOV (human ovarian carcinoma), Chinese hamster ovary cells, human foreskin fibroblasts, mouse BALB-C2Y fibroblasts, and Shionogi mouse breast carcinoma cells as determined by enumeration of cells and incorporation of [3H]thymidine. For example, Shionogi cells multiplied from $1.0 \times 10^5$ /dish to $1.0 \times 10^6$ /dish in 96 h. Cells incubated in BB-94 (156 μg/ml, a concentration in excess of its solubility) grew to the same density during this time. The HU and LA xenografts quickly lose viability in tissue culture, and so it was not possible to assess any direct cytotoxic effects of BB-94 against these cells in vitro.

Effect of BB-94 on Tumor Burden and Survival. The tumor burden of mice treated with vehicle alone was 156 ± 103 mg (mean ± SD, 8 separate determinations) 7 days after introduction of the tumor, and this had more than doubled to 542 ± 307 mg (n = 8) by 14 days. Limited survival in mice treated with vehicle alone beyond 14 days prevented reliable quantitation. However, in BB-94-treated animals the tumor burden was low at 7 days (46.4 ± 16.3 mg, n = 8) compared to animals treated with vehicle only (P < 0.001) and did not increase significantly during the first 28 days after introduction of the xenograft (61.8 ± 19.0 mg, n = 8, at 28 days) (Fig. 3).

This decreased tumor burden was associated with a dramatic increase in survival. When BB-94 was given daily 3–21 days after introduction of the xenograft, the median survival was increased from 19 days (vehicle only) to 129 days (BB-94-treated) (Fig. 4A) (P < 0.001). When BB-94 was given from days 7–21, survival was still greatly increased (median survival of vehicle-only group, 18 days vs. 129 days).
days; median survival of BB-94-treated group, 105 days; P < 0.001) (fig 4B). BB-94-treated mice eventually died from abdominal swelling caused by an accumulation of solid tumor and ascites.

Although in most experiments mice were treated daily with BB-94 for 11 to 18 days, a single dose of the drug given on day 3 was effective at resolving ascites, decreasing tumor burden, and increasing survival (Table 1). The effect of BB-94 on survival was also dose dependent. When a single dose of 40 mg/kg was given on day 3 after introduction of HU xenograft 4 of 4 mice were still alive at day 45, when treated with 30 mg/kg 3 of 4 mice were alive at 45 days, while in the group treated with 10 mg/kg 3 of 4 mice were dead by day 22. Four of four mice treated with diluent control died on day 18 (Table 1).

**Histology.** The HU and LA xenografts consisted of small clumps of tumor cells floating in mucin (Fig. 5a and c). In both xenografts the transition from ascites to solid tumor was associated with encapsulation of the clumps of tumor cells by host cells. In the case of the HU xenograft, the solid tumor which resulted from treatment with BB-94 had a relatively high proportion of stroma to tumor cells (Fig. 5b). Isolated clumps of tumor cells were visible in the stroma, and some of these appeared to be necrotic (Fig. 5b). A similar transition occurred when mice bearing the LA xenograft were treated with BB-94 (Fig. 5d). The proportion of tumor cells to stroma was much higher in LA solid tumors than in HU tumours, but some necrosis was visible toward the center of the tumor (Fig. 5d).

**Involvement of Matrix Metalloproteinase.** Further experiments showed that the observed antitumor effects of BB-94 were dependent upon its MMP inhibiting properties and not to some other nonspecific mechanism. A diastereoisomer of BB-94, BB-1268, which is 670-fold less potent an inhibitor of collagenase than BB-94, had no effect on the tumor biology of the HU xenograft. Animals treated with BB-1268 developed ascites in a manner identical to those treated with vehicle alone.

Inhibition of MMP activity in the peritoneal cavity of the host caused a transition from ascites to solid tumor probably by shifting the balance from matrix breakdown to matrix synthesis. We examined by zymography the MMP activity in HU-bearing mice treated with BB-94 or vehicle alone. Inactive proforms of the $M_1$, 92,000 and $M_1$, 72,000 enzymes were detected in ascites from mice treated with vehicle alone, but no activated forms of these enzymes could be detected. It was not possible to determine collagenase or stromelysin expression by this technique due to lack of sensitivity. Ascites from xenograft-bearing mice is extremely thick and mucinous, and this limits the amount of material which can be loaded onto polyacrylamide gels. After treatment with BB-94, ascites resolves and the large amounts of mucin are no longer present, enabling larger amounts of material to be loaded onto gels. When 80 µg of solid tumor from a BB-94-treated, HU-bearing mouse were analyzed by gel substrate analysis, activated $M_1$, 92,000 enzyme was detected in addition to the inactive forms of the $M_1$, 92,000 and $M_1$, 72,000 enzymes (Fig. 6).

We propose that activity of MMP in HU-bearing mice keeps the tumor in the ascitic phase and prevents the laying down of extracellular matrix proteins and the formation of solid tumor. Consequently, when these enzymes are inhibited by BB-94, stroma formation is favored.

**DISCUSSION**

Metastasis of ovarian carcinoma outside the peritoneal cavity is relatively rare, and death usually results from the bulk of tumor contained in this area. Relapse-free survival appears to be dependent on thorough debulking of tumor from the peritoneal cavity (13, 14). While conventional chemotherapeutic agents may initially reduce tumor bulk in ovarian cancer, recurrence of disease inevitably occurs. Because surgical debulking is often impossible, more effective therapies are required. New targets for drug intervention need to be identified in order to develop agents which are not simply toxic but which specifically disrupt tumor spread and growth. BB-94, a broad-spectrum matrix metalloproteinase inhibitor, is potentially such an agent. We have reported here that BB-94 may be effective in preventing the growth of ovarian carcinoma, apparently by changing the interaction between tumor cells and their stroma.
Tumor cells are dispersed in stroma which they themselves induce. This stroma separates tumors from normal host tissues and is essential to tumor growth; tumors cannot grow larger than 1–2 mm in diameter without stroma and blood vessel formation (15). Thus disruption of the specific interaction between tumor cells and their stroma is a potential target for chemotherapy.

Tumor stroma consists chiefly of connective tissue, and the process of stroma formation has been described as analogous to wound healing, tumors being wounds which do not heal (16). An essential early step in stroma formation is the leakage of plasma proteins from hyperpermeable tumor vasculature and the consequent formation of a fibrin mesh (17). Mature stroma consisting of extracellular matrix proteins then replaces the fibrin mesh in a process analogous to scar formation. This mature stroma contains collagen (types I, III, V), fibrin, fibronectin, glycosaminoglycans, new blood vessels, fibroblasts, and inflammatory cells (18). Tumors and associated stroma cells also express enzymes capable of degrading extracellular matrix proteins. Several different investigators have recently demonstrated the presence of type IV collagenases (gelatinases) (19–21), interstitial collagenase (22, 23), and stromelysins (24) in a variety of tumor types. Tumor-associated stroma is clearly a dynamic structure continually undergoing remodeling. We propose that BB-94 treatment of ovarian ascites-bearing mice disrupts the balance between synthesis and lysis of extracellular matrix components by blocking the degradative pathway and thus favoring synthesis of stromal connective tissue.

In treated mice, the thick mucinous ascites resolved and was replaced by solid tumor. This tumor was atypical in that it was completely avascular. The solid tumors so formed were encapsulated by host stromal cells. Tumor burden was also much lower in treated mice, indicating that formation of solid stroma slows the rate of division of tumor cells in our model.

Treatment i.p. of HU-bearing mice with TNF also caused resolution of ascites and formation of solid tumor. However, the remaining tumor burden was much greater in mice treated with TNF (multiple tumor nodules covered the surface of the peritoneum) than with BB-94, and the solid tumor formed after treatment was heavily vascularized in contrast to the avascular tumors which were present in BB-94-treated mice. In common with BB-94, however, TNF also induced an influx.
of PMNs into the peritoneal cavity. However, this was not involved in the formation of solid tumor, since this process occurred even when influx of PMNs into the peritoneal cavity was blocked by pretreating the animals with anti-CR3 receptor (25). Also, the influx of PMNs occurred in response to TNF in mice bearing an ovarian xenograft which did not respond to TNF treatment (26). Thus influx of PMN into the peritoneal cavity following i.p. therapy is most likely a nonspecific event not involved in actions of TNF or BB-94.

BB-94 clearly caused formation of solid tumor stroma in mice bearing the HU and LA xenografts, simultaneously resolving the ascitic disease. We have shown in the case of the HU xenograft that this leads to a dramatic reduction in tumor burden and a concomitant increase in survival. This was not due to a nonspecific effect of BB-94 but to its matrix metalloproteinase inhibitory properties because the inactive diastereoisomer of BB-94, BB-1268, had no effects on xenograft biology. Further evidence that the effects of BB-94 in promoting tumor stroma formation were mediated via its metalloproteinase-inhibiting effects is shown by the presence of the active form of the M, 92,000 type IV collagenase in the HU tumors. It is also possible that stromelysin, interstitial collagenase, and other matrix metalloproteinases are also active. While our data demonstrate that BB-94 causes tumor stroma formation in ovarian carcinoma xenografts, it provides no explanation for why this should slow tumor growth. However, other investigators have also observed that the promotion of stoma slows tumor growth. For instance, treatment of melanoma-bearing mice with proline analogues which inhibit collagen formation promotes tumor growth. For instance, treatment of melanoma-bearing mice with proline analogues which inhibit collagen formation promoted the formation of spontaneous metastasis (27). In addition Hewitt et al. (28) showed that the desmoplastic response (an excessive growth of connective tissue associated with tumors) in colorectal tumors is confined to the center of the tumor and absent from the invasive edge. Formation of tumor stroma may slow growth by physically limiting the availability of nutrients to tumor cells. Alternatively, more complex mechanisms involving the interaction of tumor cells with stromal extracellular matrix and cellular components are also likely to be important.

BB-94 does not affect processes intimately involved with cell division, as do conventional cytotoxic drugs. By inhibition of matrix metalloproteinases it resolved ascitic disease and promoted the formation of solid tumor. We have examined these effects in detail on the HU xenograft and confirmed similar effects on a second xenograft, LA. We are currently establishing other ovarian carcinoma xenografts i.p. in the nude mouse with a view to examining the antitumor efficacy of BB-94 against these. BB-94 is potentially of therapeutic use in the treatment of human ascitic ovarian carcinoma.

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REFERENCES

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