Mouse Endostatin Inhibits the Formation of Lung and Liver Metastases

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ABSTRACT

Angiogenesis is required for tumor formation. Several studies have demonstrated that tumor angiogenesis is regulated by a balance between proangiogenesis and antiangiogenesis factors and that this balance varies in different organ environments. To investigate whether expression of an endostatin inhibitor by cancer cells could alter this balance and prevent tumor formation in different organ environments, we engineered stable transfectants from RenCa mouse renal carcinoma cells and SW620 human colon carcinoma cells to constitutively secrete a mouse endostatin protein with c-myc and polyhistidine (His) tags. Production and secretion of the endostatin-c-myc-His fusion protein by endostatin-transfected cells were confirmed by immunofluorescence staining and Western blot analysis. The endostatin transfectants and control transfectants, stably transfected with a control plasmid, had similar in vitro growth rates compared with their parental cell lines. Conditioned medium from endostatin-transfected cells inhibited human umbilical vein endothelial cell proliferation by 36–51% compared with conditioned medium from control cells. After inoculation into mice, flank tumors from endostatin-transfected cells were 73–91% smaller than flank tumors from control cells after 3 weeks. Inoculation of a cell mixture containing 25% endostatin-transfected cells and 75% control cells resulted in inhibition of flank tumor formation as effective as after inoculation of 100% endostatin-transfected cells. Formation of lung metastases by RenCa endostatin-transfected cells and formation of liver metastases by SW620 endostatin-transfected cells were dramatically inhibited compared with formation of metastases by control cells. These findings demonstrate that endostatin can inhibit tumor formation in different organ environments and that gene delivery of endostatin into even a minority of tumor cells may be an effective strategy to prevent progression of micrometastases to macroscopic disease.

INTRODUCTION

Paget noted in 1889 that certain tumors metastasize preferentially to specific organs and emphasized that growth of metastases is dependent upon not only the properties of the tumor cells but also upon the environment in which the tumors cells grow (1). Similarly, tumor angiogenesis, or the development of new tumor blood vessels, is critically dependent on the interaction between tumor cells and the host organ environment (2). This process is regulated by a balance between proangiogenesis and antiangiogenesis factors (3). Metastatic cells from a specific cancer may be able to establish a balance favorable for tumor angiogenesis in some organs but not in others.

Tumor angiogenesis varies in different organ environments. For example, human renal carcinoma cells implanted under the renal capsule of nude mice produce 10–20 times more bFGF mRNA than when implanted into s.c. tissue (4). Similarly, LS174T human colon carcinoma cells produce less VEGF mRNA when experimentally grown in the liver than when grown in s.c. tissue (5). In addition, the endothelial cells that form new tumor blood vessels differ according to the host organ. New tumor blood vessels in the lung originate from capillary endothelial cells (2), whereas new tumor blood vessels in the liver originate from sinusoidal endothelial cells (6) that, unlike lung capillary endothelial cells, are fenestrated and lack a basement membrane (7). Because of these differences, the influence of antiangiogenesis factors on tumor growth may vary in different host organ environments. However, few studies have examined this aspect of angiogenesis.

One of the more potent antiangiogenesis agents, endostatin, is a cleavage product consisting of the COOH-terminal 184 amino acids of collagen XVIII (8). This Mr 20,000 protein inhibits endothelial cell migration and proliferation (8–11), and induces the regression of a wide variety of tumors grown s.c. in mice (8, 10, 11). The efficacy of endostatin in organ environments other than s.c. tissue is not well established. Endostatin has been shown in two studies to inhibit the formation or growth of lung metastases (11, 12). To our knowledge, there are no published reports on the efficacy of endostatin against liver metastases.

The aim of the present study was to determine the efficacy of endostatin in preventing tumor formation in different organ environments. We engineered stable transfectants from RenCa mouse renal carcinoma cells and SW620 human colon carcinoma cells that constitutively secrete mouse endostatin and determined the effect of endostatin on tumor formation in the flank, lung, and liver.

MATERIALS AND METHODS

Plasmids. The plasmid pTBO1#8 (8) containing the cDNA for mouse endostatin was generously provided by Dr. J. Folkman (Harvard Medical School, Boston, MA). Mouse endostatin cDNA was PCR amplified using a standard protocol with pTBO1#8 as template and two oligonucleotide primers, 5'-CACGATCCCTGGAGAAAGGTCATGAAG-3'9 and 5'-AGGGATCCTTGGAGAAGAGGTCATGAAG-3'. These primers introduced KpnI and BamHI restriction sites at the 5' and 3' ends, respectively. The resulting PCR product was digested with KpnI and BamHI and ligated into pSecTag2/HygroB (Invitrogen, Carlsbad, CA). This placed the mouse endostatin cDNA downstream of the CMV promoter and murine immunoglobulin κ chain signal peptide and upstream of a c-myc epitope and polyhistidine (His) tag. This plasmid was designated pEndoSTHB. DNA sequence analysis confirmed that the mouse endostatin cDNA sequence was inserted in the proper reading frame and without mutations.

Cell Lines. The mouse renal carcinoma cell line RenCa (13) was provided by Dr. K. Okumura (Jutendo University, Tokyo, Japan). The human colon carcinoma cell line SW620 was obtained from the American Type Culture Collection (Manassas, VA). RenCa and SW620 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. HUVEC were obtained from Clonetics (San Diego, CA) and maintained in EGM-2-MV medium (Clonetics).
Fig. 1. A, construction of plasmid pEndoSTHB encoding the mouse endostatin. cDNA for mouse endostatin (Endo) was inserted into the mammalian expression plasmid pSecTag2/HygroB, which encodes the CMV promoter (CMV), murine immunoglobulin κ chain signal peptide (Igκ), c-myc epitope (myc), and polyhistidine tag (His). B, Western blot analysis of secreted mouse endostatin-c-myc-His fusion protein. Conditioned medium from RenCa control cells (RΔ0), RenCa endostatin-transfected cells (RΔE15 and RΔE17), SW620 control cells (SΔ0C1), and SW620 endostatin-transfected cells (SΔED2 and SΔED9) was concentrated, followed by detection with an anti-c-myc mAb. The size of the mouse endostatin-c-myc-His fusion protein is estimated based on peptide sequence analysis to be M₆₂,900.

Generation of Stable Transfectants That Secrete Mouse Endostatin. Endostatin transfectants were generated by transfection of pEndoSTHB into RenCa and SW620 cells using LipofectAMINE and LipofectAMINE PLUS Reagent (Life Technologies, Inc., Gaithersburg, MD) following the manufacturer’s instructions. Two days later, cells were placed under hygromycin selection at 800 μg/ml. Four weeks after transfection, hygromycin-resistant colonies were expanded and tested for endostatin-c-myc-His fusion protein production and secretion by immunofluorescence and Western blot analysis as described below. Control transfectants were generated in a similar manner, except the parent plasmid pSecTag2/HygroB (without endostatin cDNA) was substituted for pEndoSTHB.

Western Blot Analysis. One million cells were plated onto 60-mm plates and incubated for 24 h. The medium was replaced with 1 ml DMEM, and cells were incubated for 24 h. One ml of conditioned medium was concentrated in a Microcon 10 microconcentrator (Amicon, Beverly, MA) to 20 μl and subjected to electrophoresis under reducing conditions on an 18% polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) and incubated overnight in 5% nonfat milk in PBS at 4°C. After briefly washing in 1% nonfat milk and 0.1% Tween 20 in PBS, the membrane was incubated with anti-c-myc mouse mAb (Sigma Chemical Co., St. Louis, MO) diluted 1:100. After three 10-min washes in 1% nonfat milk and 0.1% Tween 20 in PBS, membranes were incubated in horseradish peroxidase-conjugated antimouse immunoglobulin (Amersham, Arlington Heights, IL) diluted 1:4000. After three 10-min washes in TBS containing 0.05% Tween 20, proteins were detected using the enhanced ECL kit (Amersham).

Immunofluorescence. Cells were grown on eight-well glass slides (Lab-Tek, Naperville, IL), fixed in 4% paraformaldehyde in PBS, and permeabilized with 0.2% Triton X-100 in PBS. After washing in PBS, cells were incubated with either 1.25 μg/ml anti-His mouse mAb (Invitrogen, Carlsbad, CA) for RenCa cells or anti-c-myc mouse mAb (Sigma) diluted 1:200 for SW620 cells for 1 h. Cells were washed in PBS and incubated in 9.3 μg/ml FITC-conjugated antimouse immunoglobulin (Biosource, Camarillo, CA) in 10% nonfat milk in PBS for 1 h. After washing in PBS, cells were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and photographed with an Axiopt fluorescence microscope (Carl Zeiss, Thornwood, NY) at ×60. Background staining using the anti-c-myc mAb on RenCa cells was too high for accurate discrimination.

In Vitro Growth Rates. Cells were plated onto 96-well plates at a density of 2000 cells/well in quadruplicate. At designated time points, the numbers of cells were quantified using a colorimetric MTT assay as described previously (14).

Endothelial Cell Proliferation Assay. Stable transfectants were plated onto 150-mm plates at a density of 5–10 million cells/plate and incubated for 48–72 h. The cells were washed with PBS, and 5 ml of DMEM were added to each plate. Cells were incubated for an additional 24 h, and 5 ml of conditioned serum-free medium were collected and concentrated to 1 ml using a Centrifu...
was designated pEndoSTHB (Fig. 1A). pEndoSTHB or pSecTag2/HygroB was transfected into RenCa and SW620 cells. Two endostatin-transfected RenCa clones (RΔE15 and RΔE17) that secreted the expected M, 29,000 protein and one control clone (RΔ0) were selected (Fig. 1B). Similarly for the SW620 cells, two endostatin-transfected clones (SΔED2 and SΔED9) and one control clone (SΔ0C1) were selected.

Stable transfectants were examined for endostatin expression by immunofluorescence. Endostatin-secreting RenCa clones (Fig. 2b) and SW620 clones (Fig. 2d) demonstrated cytoplasmic staining consistent with the presence of the endostatin-c-myc-His fusion protein prior to secretion. The control RenCa clone (Fig. 2a) and control SW620 clone (Fig. 2c) showed only minimal background staining.

**In Vitro Growth Rates of Stable Transfectants.** Cells were seeded onto 96-well plates, and the number of cells was quantified at specific time points by MTT assay. RenCa stable transfectants and parental RenCa cells grew at similar rates in vitro (Fig. 3A). In addition, SW620 stable transfectants and parental SW620 cells displayed similar in vitro growth rates (Fig. 3B). Thus, endostatin secreted into the culture medium by endostatin-expressing clones had no inhibitory effect on the proliferation of these nonendothelial cells.

**Mouse Endostatin Inhibits Endothelial Cell Proliferation.** The biological activity of secreted mouse endostatin-c-myc-His fusion protein was tested against HUVEC cells. Conditioned medium from RΔ0 control cells did not inhibit HUVEC cell growth as compared with DMEM alone (Fig. 4). Conditioned medium from RΔE15 and RΔE17 endostatin-transfected cells inhibited HUVEC proliferation by 51 and 40%, respectively, compared with conditioned medium from RΔ0 control cells. Similar results were obtained for conditioned medium from SW620 stable transfectants, with conditioned medium from SΔED2 and SΔED9 endostatin-transfected cells inhibiting HUVEC proliferation by 39 and 36%, respectively, compared with conditioned medium from SΔ0C1 control cells.

**Mouse Endostatin Inhibits Formation of s.c. Tumors.** To determine whether endostatin secreted from RenCa cells inhibited s.c. tumor formation, 5 × 10^6 cells from each RenCa clone were injected s.c. into the flanks of immune-competent BALB/c mice. Similarly, 5 × 10^6 cells from each SW620 clone were injected s.c. into the flanks of nude BALB/c mice. For RenCa clones, RΔ0 control cells formed tumors rapidly, but RΔE15 and RΔE17 endostatin-transfected
Mouse Endostatin Inhibits Formation of Lung Metastases. To investigate the effect of endostatin on tumor formation in the lung, we injected $5 \times 10^5$ cells from each RenCa clone into the tail veins of BALB/c mice. The parental RenCa cells in this well-established model produce abundant lung metastases after 4 weeks (15). After sacrificing mice at 4 weeks, lungs from mice injected with RΔE15 and RΔE17 endostatin-transfected cells had few or no metastases, whereas lungs in mice injected with control cells had metastases that were too numerous to count (Fig. 6A; Table 1). Lungs from mice injected with RΔ0 cells also weighed significantly more than lungs from mice injected with RΔE15 and RΔE17 cells. This experiment was repeated in 15 more mice with similar results (data not shown).

We found previously that delayed flank tumor growth after inoculation of RΔ0 control cells was 73 and 84% smaller, respectively, compared with RΔ0 tumors after 21 days (Fig. 5A). SΔ0C1 control cells also formed tumors rapidly, whereas SΔED2 and SΔED9 endostatin-transfected cells formed tumors that were 91 and 82% smaller, respectively, after 21 days (Fig. 5B). Mice bearing RΔ0 and SΔ0C1 tumors were euthanized on day 21 because of tumor burden. Mice bearing RΔE15, RΔE17, SΔED2, and SΔED9 tumors were followed after 21 days, and their tumors eventually grew at an appreciable rate (data not shown). Seven to 8 weeks after initial tumor inoculation, mice with tumors from endostatin-transfected cells were euthanized because of tumor burden.

Cell mixing experiments were performed to determine whether endostatin secretion by a subpopulation of cells could inhibit the growth of the remaining population of cells. SΔ0C1 control cells and SΔED9 endostatin-transfected cells were mixed in various ratios before s.c. implantation into nude mice. Tumor growth was equally inhibited, independent of whether 100, 75, or 25% of the cells expressed endostatin (Fig. 5C).

In a separate experiment, each clone was injected s.c. into mice to generate tumors for immunohistochemical analysis. During the first 3 weeks after inoculation, flank tumors from endostatin-transfected cells were too small to harvest and section; therefore, tumors from endostatin-transfected cells were harvested between 3 and 5 weeks after inoculation, when they had grown beyond 2–3 mm in diameter. No immunohistochemical staining for endostatin-c-myc–His fusion protein was observed in any of the sections, suggesting down-regulation or loss of the endostatin transgene by the time these tumors had grown several mm in diameter (data not shown). Sections were also stained for tumor blood vessels using an anti-CD31 mAb. Blood vessel densities were determined and were not significantly different between tumors from endostatin-transfected clones and control clones (data not shown).

Mouse Endostatin Inhibits Formation of Lung Metastases. To investigate the effect of endostatin on tumor formation in the lung, we injected $5 \times 10^5$ cells from each RenCa clone into the tail veins of BALB/c mice. The parental RenCa cells in this well-established model produce abundant lung metastases after 4 weeks (15). After sacrificing mice at 4 weeks, lungs from mice injected with RΔE15 and RΔE17 endostatin-transfected cells had few or no metastases, whereas lungs in mice injected with control cells had metastases that were too numerous to count (Fig. 6A; Table 1). Lungs from mice injected with RΔ0 cells also weighed significantly more than lungs from mice injected with RΔE15 and RΔE17 cells. This experiment was repeated in 15 more mice with similar results (data not shown).

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ulation of endostatin-transfected cells was associated with loss of endostatin expression as assessed by immunohistochemistry. To test whether pulmonary metastasis formation by endostatin-transfected cells was associated with loss of endostatin biological activity, lung metastases generated from endostatin-transfected cells (RΔE15 and RΔE17) and control cells (RΔ0) were harvested and implanted s.c. into BALB/c mice. Although we had demonstrated previously that RΔE15 and RΔE17 endostatin-transfected cells grew poorly in the flank compared with RΔ0 control cells after s.c. inoculation of a cell suspension (Fig. 5A), we observed different results after s.c. implantation of harvested pulmonary metastases. There was no significant difference in the size of flank tumors grown from RΔ0 pulmonary metastases compared with those grown from either RΔE15 or RΔE17 pulmonary metastases (Fig. 6B). These data are consistent with down-regulation or loss of expression as an explanation for eventual tumor formation by endostatin-transfected cells.

**Mouse Endostatin Prevents Formation of Liver Metastases.** We next examined the effect of endostatin expression on formation of experimental liver metastases. Others have demonstrated previously that SW620 cells injected into the spleens of nude mice generate experimental liver metastases in 60% of mice after 3 months (16). We injected 5 × 10⁶ cells from each SW620 stable transfectant into the spleens of nude mice. After 12 weeks, the mice were sacrificed, and their livers were harvested. Three of the five livers from mice injected with SΔOCl control cells developed liver metastases, whereas none of the 10 livers from mice injected with SΔED2 or SΔED9 endostatin-transfected cells developed liver metastases (Fig. 7A; Table 2). This experiment was repeated in 15 more mice with similar results (data not shown).

**DISCUSSION**

We have demonstrated in two different cancers, mouse renal carcinoma and human colon carcinoma, that secretion of mouse endostatin from these cancer cells inhibits tumor formation in three different organ environments, s.c. tissue, lung, and liver. Other studies have shown that endostatin can inhibit lung metastases (11, 12), but to our knowledge, this is the first study to demonstrate that endostatin is effective in the liver environment.

These results demonstrate the ability of endostatin to inhibit tumor or metastasis formation in different organ environments and do not examine the efficacy of endostatin against established tumors. A recent study by Bergers et al. (17) found that in a transgenic mouse model of spontaneous β-islet cell tumors, endostatin was highly effective in preventing progression of hyperplastic nodules to small tumors but was ineffective in inhibiting the growth of established invasive carcinomas. Achieving regression of established tumors is a more difficult undertaking than inhibiting tumor formation. Endostatin has been demonstrated to induce regression of tumors grown s.c. on the flanks of mice (8, 10, 11), but actual regression, as opposed to growth inhibition, has not been demonstrated in the lung or liver environments.

Gene transfer of endostatin and other angiogenesis inhibitors has been performed by other groups. Nguyen et al. (9) reported the use of an adeno-associated virus to transduce tumor cells with the endostatin gene in vitro and demonstrated that the secreted endostatin inhibits endothelial cell proliferation. In another study, plasmid DNA containing the endostatin cDNA was injected i.m. into mice and resulted in the inhibition of s.c. tumors and spontaneous pulmonary metastases (11). In addition, s.c. inoculation of fibrosarcoma cells stably transfected with the gene for another angiogenesis inhibitor, angiostatin, resulted in inhibition of flank tumor growth and spontaneous pulmonary metastases (18).

For the purposes of this study, stable transfection of the endostatin gene into cancer cells, followed by inoculation of these cancer cells into mice, avoided some potential problems associated with systemic administration of endostatin. Production of large amounts of biologically active

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**Table 1** Formation of lung metastases from RenCa stable clones

<table>
<thead>
<tr>
<th>RenCa clone</th>
<th>Mean number of metastases (range)</th>
<th>Mean weight (g) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>RΔ0</td>
<td>&gt;100</td>
<td>1.38 ± 0.36</td>
</tr>
<tr>
<td>RΔE15</td>
<td>11–33 (18.4)</td>
<td>0.67 ± 0.06⁸</td>
</tr>
<tr>
<td>RΔE17</td>
<td>0–15 (3.0)</td>
<td>0.73 ± 0.13</td>
</tr>
</tbody>
</table>

* A single-cell suspension (5 × 10⁶ cells in 100 μl of HBSS) was injected into the tail veins of BALB/c mice (n = 5/group). Mice were sacrificed after 1 month. This is one representative experiment of two.

*⁸ P < 0.01, in comparison with RΔ0.

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**Table 2** Formation of liver metastases from SW620 stable clones

<table>
<thead>
<tr>
<th>SW620 clone</th>
<th>No. of livers with metastases/ Total number of livers</th>
<th>Mean weight (g) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>SΔOCl</td>
<td>3/5</td>
<td>3.07 ± 1.15</td>
</tr>
<tr>
<td>SΔED2</td>
<td>0/5</td>
<td>2.14 ± 0.29⁸</td>
</tr>
<tr>
<td>SΔED9</td>
<td>0/5</td>
<td>2.36 ± 0.26</td>
</tr>
</tbody>
</table>

* A single-cell suspension (5 × 10⁶ cells in 100 μl of HBSS) was injected into the spleens of athymic BALB/c mice (n = 5/group). Mice were sacrificed after 3 months.

*⁸ P = 0.12, in comparison with SΔOCl.

*⁻² P = 0.22, in comparison with SΔOCl.
recombinant endostatin to treat cohorts of mice can be difficult. In addition, the pharmacokinetics of endostatin absorption, distribution, and elimination after systemic administration is unknown and may result in different tissue levels in various organs, particularly in the liver, which is the primary site of metabolism of many drugs (19). Although host organ environment may have influenced the expression of the endostatin gene from our endostatin-transfected clones, expression was enough at all sites tested to inhibit tumor formation.

Results of immunohistochemical staining studies of flank tumors indicated that endostatin expression was no longer detectable once tumors had grown beyond a small size. Endostatin expression may have been down-regulated or lost after tumor cell implantation into mice in the absence of hygromycin selection. Alternatively, proangiogenesis factors such as bFGF and VEGF may have eventually been up-regulated in these tumors, overcoming the effects of endostatin.

After flank inoculation of a cell mixture consisting of only 25% of endostatin-transfected cells, flank tumor growth was inhibited as much as when the inoculum consisted of 100% endostatin-transfected cells. However, we also observed that lung metastases from endostatin-transfected cells, after implantation into the flank, formed flank tumors as rapidly as lung metastases from control cells. These results may be consistent because an endostatin-expressing cell line likely represents a heterogeneous population in which some cells express little or no endostatin. When this heterogeneous population is implanted s.c., endostatin secreted by the majority of cells appears to prevent tumor formation by any immediately adjacent cells that do not express endostatin. In contrast, when inoculated i.v. as a single-cell suspension, individual cells that express little or no endostatin may successfully establish pulmonary metastases. This scenario presumes that the endostatin-producing cells that lay dormant in the lung do not produce levels of endostatin high enough to prevent metastasis formation elsewhere in the lung.

The observed reduction in tumor growth associated with endostatin secretion was most likely attributable to the biological activity of the fusion protein and not secondary to other causes. Clonal selection for a less tumorigenic phenotype is hypothetically possible but improbable for several reasons: (a) we used two different carcinoma cell lines and selected two endostatin-expressing clones from each cell line. We also selected hygromycin-resistant control clones that had been transfected with the same expression vector without the endostatin cDNA insert. The statistical likelihood that only the four clones of the six that we selected are incapable of in vivo growth for reasons unrelated to endostatin expression is exceedingly low; (b) we demonstrated that the expressed endostatin was biologically active against endothelial cells in vitro; (c) the control clones were clearly tumorigenic, yet implantation of a mixture of cells in which only 25% of cells expressed endostatin inhibited growth of the control cells. Host-immune response to the endostatin-c-myc-His fusion protein is also an unlikely cause of the observed results because significant antitumor effects were observed in both immune-competent hosts and congenitally athymic hosts. Ideally, we would have liked to perform experiments using endostatin-neutralizing antibodies. However, the absence of an endostatin antibody capable of neutralizing all of its biological activity precluded such an experiment. The anti-c-myc and anti-His mAbs reacted with the recombinant fusion protein but did not attenuate the ability of endostatin to inhibit HUVEC proliferation in vitro (data not shown). Accordingly, these antibodies could not be used for neutralization experiments.

Many issues regarding endostatin and other angiogenesis inhibitors remain unanswered. The molecular mechanisms by which endostatin exerts its effects are not well understood. Recently, Knebelmann et al. (20) reported that endostatin inhibits activation of mitogen-activated protein kinase, which is a downstream target in the bFGF and VEGF signaling pathways. Examination of the effect of endostatin on these pathways in different environments may shed more light on organ-specific differences in endostatin function. One drawback to gene transfer of endostatin into cancer cells prior to inoculation into mice is that this strategy did not allow us to study the efficacy of endostatin against established tumors in different organ environments. We are currently examining the efficacy of endostatin against established tumors in various organ environments using baculoviral and herpes simplex viral vectors to deliver the endostatin gene.

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