Genistein Regulation of Transforming Growth Factor-α, Epidermal Growth Factor (EGF), and EGF Receptor Expression in the Rat Uterus and Vagina

Nadine M. Brown and Coral A. Lamartiniere

Department of Pharmacology and Toxicology, and University of Alabama at Birmingham Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, Alabama 35294

Abstract
Epidemiological reports and laboratory data have associated soy and genistein with reduced incidence of uterine, breast, and prostate cancers, cardiovascular disease and osteoporosis, and lower total blood cholesterol. The aim of this study was to investigate the effect of genistein in the uterus and vagina of rats, focusing our attention on the distribution of transforming growth factor (TGF) α, epidermal growth factor (EGF), and EGF receptor. A pharmacological dose of genistein (500 μg/g body weight) injected in rats on days 16, 18, and 20 postpartum resulted in significant uterine wet weight gain, with hypertrophy of the luminal and glandular epithelium of the uteri, and squamous epithelium of the vagina in 21-day-old animals. At 50 days of age, hypertrophy was no longer evident in the uterus and vagina. Prepubertal genistein treatment resulted in significantly increased EGF immunostaining in individual stromal cells and reduced EGF receptor immunostaining in blood vessels of the uterus. Genistein-treated rats had decreased TGF-α immunostaining in glandular and luminal epithelium and a slight increase in EGF receptor immunostaining in stromal cells of the uterus. This suggests paracrine interaction between cells elevating the level of EGF ligand in the stroma and the EGF receptor in the luminal and glandular epithelium, resulting in uterine hypertrophy. In the vagina, genistein did not cause significant alterations to the EGF-signaling pathway in 21- and 50-day-old rats. We conclude that pharmacological doses of genistein during the prepubertal period can modulate the EGF-signaling pathway in the uterus and exert a uterotrophic response in a short-term manner.

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Introduction
Soy products have received much attention as dietary components to promote better health. Epidemiological reports and laboratory data have associated soy with reduced incidence of endometrial, breast, and prostate cancers, cardiovascular disease and osteoporosis, and lower total blood cholesterol (1–3). The most abundant isoflavone component of soy is genistein. In vitro genistein has been reported to possess estrogen-like actions, to inhibit protein tyrosine kinases and topoisomerase II, and to inhibit cell growth at high concentrations (reviewed in Ref. 4). In the prepubertal rat mammary gland, pharmacological, but not physiological, doses of genistein initially result in measurable up-regulation of EGF receptor expression and enhance mammary gland development (5, 6). These actions result in cell differentiation and subsequent reduced expression of the EGF receptor in mammary terminal end buds. We speculate that these genistein actions suppress the development of chemically induced mammary cancer in rats (4–7). In the rat dorsolateral prostate, genistein can inhibit EGF receptor expression (8). This direct action of genistein may play a role in regulating cell proliferation and protecting against prostate cancer.

Other than knowing that pharmacological doses of genistein are uterotrophic (9, 10), little is known about the genistein mechanism of action in the uterus. The uterus is a complex tissue comprised of circular and longitudinal muscle, endometrial stroma, and luminal and glandular epithelium, the regulation of which has been shown to be controlled by several steroid- and growth factor-signaling pathways. An additional issue is the potential of estrogendependently active chemicals to exert developmental alterations to the reproductive tract, in particular to the uterus and vagina. Estrogen exposure during the neonatal period has been reported to cause persistent vaginal cornification in adult mice (11). Furthermore, prenatal exposure of human females and mice to diethylstilbestrol has been reported to result in vaginal adenocarcinoma (12, 13).

The aim of this study was to compare the distribution of TGFα, EGF, and EGF receptor in uterus and vagina of the immature and young adult female rats exposed prepubertally to genistein by using immunohistochemistry. Pharmacological doses of genistein were used because dietary physiological doses did not manifest measurable changes in these biomarkers. We investigated these distributions 30 days after the last genistein treatment to determine whether prepubertal genistein treatment would have a prolonged effect on this pathway in these tissues.

1 The abbreviations used are: TGF, transforming growth factor; EGF, epidermal growth factor.
Results

Morphometric Alterations in the Uterus. Injections of genistein to prepubertal female rats resulted in a 3.5 greater uterine wet weight and no difference in body weight in 21-day-old female rats (Table 1). Histomorphological evaluation of the uteri in the 21-day-old rats treated prepubertally with genistein showed evidence of epithelial hypertrophy associated with early estrogenic stimulation (Fig. 1). This was true for both the luminal epithelium that lines the uterine cavity and the glandular epithelium. The longitudinal and circular muscle and stroma were normal in appearance. At 50 days of age, the body and uterine wet weights did not differ for rats treated prepubertally with genistein and vehicle. There were no apparent histopathological lesions in uteri of 21- or 50-day-old rats treated prepubertally with genistein.

Table 1  Body and uterine weights in female rats treated prepubertally with genistein and DMSO

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age</th>
<th>Body weight (g)</th>
<th>Uterine weight (mg)</th>
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</thead>
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<tr>
<td>DMSO</td>
<td>21</td>
<td>50 ± 1</td>
<td>31 ± 2</td>
</tr>
<tr>
<td>Genistein</td>
<td>21</td>
<td>50 ± 1</td>
<td>110 ± 11*</td>
</tr>
<tr>
<td>DMSO</td>
<td>50</td>
<td>217 ± 8</td>
<td>295 ± 34</td>
</tr>
<tr>
<td>Genistein</td>
<td>50</td>
<td>196 ± 5</td>
<td>296 ± 33</td>
</tr>
</tbody>
</table>

*P < 0.001 compared with DMSO-treated animals.

EGF Immunohistochemistry in the Uterus. Immuno-histochemical evaluation for TGF-α in uteri of 21-day-old vehicle-treated rats revealed its expression to produce strong (+ + +) staining in the glandular epithelium and moderate (+ +) staining in luminal epithelium (Table 2 and Fig. 1A). However, in genistein-treated animals, TGF-α was light (+) in both glandular and luminal epithelium (Table 2 and Fig. 1B). In stroma, and longitudinal and circular muscle of both vehicle and genistein-treated rats, TGF-α staining was absent (0) to light (+).

The intensity of TGF-α in uterine glandular epithelium of 50-day-old as compared with 21-day-old control animals diminished slightly and was moderate (+ +) in both glandular and luminal epithelium of control and genistein-exposed tissue. Stromal tissue and circular and longitudinal muscle of the uterus of DMSO- and genistein-treated rats showed light (+) TGF-α staining. Blood vessels throughout the uterus of 50-day-old rats also showed light (+) stain in the tunica intimad and tunica media, but moderate (+ +) staining was present in plasma and RBCs of control rats, whereas the plasma and RBCs of genistein-treated rats showed only light staining (+).

EGF Immunohistochemistry in the Uterus. The two most striking differences between tissues from control and genistein-treated rats were evident for the immunohisto-staining of EGF in the stroma and blood vessels of 21-day-old rats. In uteri from genistein-treated rats, there was a unique intense (+ + + +) staining of individual cells scattered throughout the stroma (Table 2 and Fig. 1D). This staining was consistently present in juxtaposition to the nucleus and was not seen in control rats (Fig. 1C). In comparison to uterine epithelial cells of control animals, the stromal cells did not stain or had light (+) staining only. No difference was observed in EGF staining of glandular or luminal epithelium of control and genistein-treated rats; both stained moderately (+ +). In genistein-treated animals, the glandular epithelial staining had a grainy appearance. Epithelial cells that lined the blood vessels and some of the RBCs stained intensely (+ + + +) in control tissue, but this staining was absent or light (+) in genistein-treated animals. EGF staining in the circular muscle of control uterus was moderate (+ +) diffuse staining, whereas uteri from genistein-treated rats had strong (+ + +) grainy staining. Longitudinal muscle of control uterus had light (+) diffuse staining, whereas uteruses from genistein-treated rats was moderate (+ +) grainy staining.

At 50 days postpartum, as was true at 21 days of age, there was no difference in EGF staining of the glandular or luminal epithelium; both stained moderately (+ +) in vehicle- and genistein-treated rats. In general, the stroma lacked staining or stained lightly (+) in both control and genistein-treated tissue. The most striking change seen at this age was the restoration of EGF staining in the tunica intima and tunica media of blood vessels of genistein-treated rats. The distinctive staining seen in individual stromal cells at 21 days of age was not as pronounced at this age, but it could be identified. Circular muscle had strong (+ + +) and longitudinal muscle had moderate (+ +) EGF staining in uteri of DMSO and genistein-treated rats. The grainy character of the staining seen in genistein-treated rats at 21 days was not pronounced at this age.

EGF Receptor Immunohistochemistry in the Uterus. Immunohistochemical evaluation for EGF receptor in uteri of DMSO and genistein-treated, 21-day-old rats revealed moderate (+ +) staining in glandular and luminal epithelia (Table 2 and Fig. 1, E and F). Overall, in the stroma of DMSO and genistein-treated uteri, the EGF receptor staining was absent (0) to light (+), whereas in individual cells of the stroma, there was moderate (+ +) and strong (+ + +) EGF receptor staining in control and genistein-treated rat uteri, respectively. The intense staining of blood vessels for EGF was not seen in EGF receptor-stained tissue. The blood vessels stained lightly (+), and with few exceptions, there was no staining evident in blood plasma or RBCs. EGF receptor staining was light (+) in circular and longitudinal muscle of vehicle and genistein-treated rats.

At day 50 postpartum, glandular and luminal epithelium stained moderately (+ +) in uteri of vehicle and genistein-treated rats. In stroma cells and blood vessel tunica, plasma and cell staining was absent (0) to light (+) in uteri of vehicle and genistein-treated rats. In the individual cells of the stroma, genistein resulted in moderate EGF receptor staining as compared with light (+) staining in control uteri. Circular and longitudinal muscle of uterine from vehicle and genistein-treated rats had no staining (0) or light (+) staining.

Vaginal Histomorphology and Immunohistochemical Staining. Vaginal epithelium of genistein-treated, 21-day-old rats showed squamous epithelial that was 9–10 cell layers thick as compared with 3–4 cell layers in thickness in control animals (Table 3). At 50 days of age, the hypertrophy
induced by prepubertal genistein treatment was no longer evident. In both groups, the thickness of the vaginal epithelial lining fluctuated with the stage of the estrous cycle. No histopathological lesions were observed in the vagina of the vehicle and genistein-treated animals at 21 or at 50 days of age.

TGF-α immunostaining was moderate (++) and similar in vaginal epithelium of control and genistein-treated, 21-day-old rats. By day 50 postpartum, TGF-α staining was moderate to strong in both groups of animals.

EGF staining was moderate (++) in the epithelial layer of the vagina of both vehicle and genistein-treated animals. However, in control tissue, the third cell layer of the stratified squamous epithelium was defined as a distinct line of cells by strong (++++) EGF staining. This staining was not seen in genistein-treated animals. The intense EGF staining seen in the third layer of vaginal squamous epithelial cells of 21-day-old control tissue was not observed at 50 days of age, and no difference in EGF intensity staining was observed from the prepubertal genistein treatment at this age.

Fig. 1. Immunohistochemical staining for EGF, TGF-α, and EGF receptor in rat uteri. Photographs were taken from uteri of 21-day-old rats injected s.c. with DMSO (A, C, and E) or 500 µg genistein/g body weight (B, D, and F) on days 16, 18, and 20 postpartum. Note brown immunostaining for TGF-α in A and B; EGF in C and D; and for EGF receptor in E and F. BV, blood vessel; CM, circular muscle; GE, glandular epithelium; ISC, individual stromal cell; LM, longitudinal muscle; LE, luminal epithelium; S, stroma. ×190.
EGF receptor staining was present in equal intensity (+++) in vaginal epithelium of vehicle and genistein-treated rats at 21 and 50 days of age. The vessels stained lightly (+) for EGF receptor, and with few exceptions, there was no staining evident in blood plasma or RBCs.

**Discussion**

This report confirms the uterotrophic action (5, 6, 10) of genistein and demonstrates its ability to modulate the EGF signaling pathway in the rat uterus. Pharmacological doses of genistein administered to prepubertal rats resulted in a significant uterine wet weight gain and with hypertrophy of the luminal and glandular epithelium. This uterotrophic effect was not evident in 50-day-old rats. We have reported previously that prepubertal genistein treatment did not significantly alter oocyte/follicular counts and numbers of atretic follicles and corpora lutea, or circulating estradiol and progesterone in 50-day-old rats (4, 6). Likewise, we have demonstrated that perinatal exposure to physiological concentrations of genistein in the diet did not result in significant alterations to the reproductive tract of F1 females (7).

The EGF-signaling pathway has been implicated in uterine and vaginal development, particularly as mediators of estrogen action. With estrogen, uterine EGF receptor transcripts were elevated in ovariec-tomized rats (14) within 3 h of estrogen injection, remained elevated at 6 h after estradiol injection, and then declined thereafter. Our immunohistochemical analysis revealed that TGF-α, EGF, and EGF receptor immunoreactivities were located in all major cell types of the rat uterus. This confirms the report of Lin et al. (15) in rats and Chegini et al. (16, 17) in humans. In contrast, the location of EGF-signaling proteins in mice is unresolved (18–20).

In the control rat uterus, TGF-α immunoreactivity was most intense in the glandular epithelium and luminal epithelium. However, genistein treatment decreased the TGF-α immunostaining intensity in the glandular and luminal epithelium of 21-day-old rats. As the animals matured to day 50 postpartum and the genistein concentrations decreased, the genistein effect on TGF-α was no longer evident in all compartments. This suggests that the effect of genistein on TGF-α in the uterus is direct and only in the presence of genistein.

The most dramatic effect of genistein was on EGF expression. Genistein treatment resulted in significantly increased EGF immunostaining in certain individual cells of the uterine stroma, demonstrating selective induction of EGF in 21-day-old rats. On the other hand, EGF immunostaining in the blood vessel tunica, plasma, and cells was significantly reduced after genistein treatment. Genistein down-regulation of EGF expression in blood vessels is consistent with its possessing antiangiogenic and chemopreventive properties in the breast (7, 21). No significant change was observed in the luminal and glandular epithelium. In 50-day-old animals, prepubertal genistein treatment did not result in persistent alterations to EGF expression.

EGF receptor expression in the uteri of 21-day-old rats was predominately in the luminal and glandular epithelium and in individual cells of the stroma. Prepubertal genistein treatment did not significantly alter EGF receptor immuno-

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**Table 2** Immunohistochemical localization of TGF-α, EGF, and EGF receptor in uteri of rats treated prepubertally with genistein or DMSO

Female Sprague Dawley rats received injections s.c. with 500 μg of genistein/g body weight or an equivalent volume of DMSO on days 16, 18, and 20 postpartum. Immunostaining was classified as "not detectable" (0), "detectable" (+), "moderate" (++) or "intense" (+++), or "intense" (++++) (Ref. 28). Values represent the mean for six rats/group.

<table>
<thead>
<tr>
<th>Location</th>
<th>TGF-α Genistein</th>
<th>EGF Genistein</th>
<th>EGF receptor Genistein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uteri from 21-day-old rats</td>
<td>DMSO</td>
<td>Genistein</td>
<td>DMSO</td>
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<tr>
<td>Epithelium</td>
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<td>Luminal</td>
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<tr>
<td>Individual stromal cells</td>
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<td>0</td>
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<tr>
<td>Blood vessel tunica</td>
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<td>+</td>
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<td>Blood plasma and cells</td>
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<tr>
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<tr>
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<td>0/+</td>
<td>+</td>
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<td>Uteri from 50-day-old rats</td>
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<td>Stroma</td>
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<tr>
<td>Overall stroma</td>
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<tr>
<td>Blood vessel tunica</td>
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<td>Muscle layers</td>
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<tr>
<td>Circular</td>
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<td>++</td>
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</tr>
<tr>
<td>Longitudinal</td>
<td>++</td>
<td>++</td>
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</table>
staining intensity in any compartment of 21- or 50-day-old rat uterus. The presence of the EGF receptor and its ligands in the luminal and glandular epithelium can account for the hypertrophy, presumably from phosphorylation of the receptor, an action that is difficult to measure in vivo, especially by immunohistochemical techniques.

Although TGF-α, EGF, and EGFR receptor immunostaining was detected in all major cell types of the vagina, genistein was not found to alter their regulation in this tissue. An enhanced proliferative response in the vagina with lack of significant increase in the EGF signaling pathway suggests differential mechanisms of action in vagina and uterus. Although genistein may be acting via the estrogen receptor and EGFR receptor pathway in the uterus, in the vagina it may act independently of the EGF-signaling pathway. That genistein did not result in histopathological lesions in the vagina is viewed as reduced potential for genistein to cause toxicity in this organ.

Data strongly suggest that estrogen induction of physiological processes, including proliferation and differentiation, are mediated by EGF-EGFR receptor interaction (22–24). Conceivably, activation resulting from EGF binding to transmembrane-bound EGFR receptor leads to its autophosphorylation and activation of its tyrosine kinase activity and proliferation in target cells. Previous studies have shown that estrogen, like genistein, induces cell proliferation primarily in the epithelium. What is interesting about genistein in the rat uterus is that increased cell proliferation in the epithelial cells after prepubertal genistein treatment is not associated with increased EGFR receptor expression in the luminal and glandular epithelium but with increased expression of the EGF ligand in the surrounding stromal cells. This suggests a paracrine mechanism involving the elevated level of EGF ligand in the stroma and the EGFR receptor in the luminal and glandular epithelium (25).

Materials and Methods

Animals and Chemicals. Female Sprague Dawley CD rats (Charles River Breeding Laboratories, Raleigh, NC) were bred in the University of Alabama at Birmingham Animal Resources Facility. Dams were fed Prolab 3000 animal diet (Agway, Inc., Syracuse, NY) until parturition and then transferred to the American Institute of Nutrition-76A diet (Harlan Teklad, Madison, WI), a semipurified diet containing no detectable phytoestrogens (limit of detection, 10 nmol/l). Diet and water were consumed ad libitum in a climate-controlled room with a 12-h light/12-h dark cycle. At birth, litters were reduced so that each dam had 10 offspring (4–6 females/dam). For genistein treatment, one-half of the female offspring from each litter received 500 μg of genistein/g body weight via s.c. injections; the other half received an equivalent volume of the vehicle, DMSO, only on days 16, 18, and 20 postpartum. Genistein was purified (26) from a concentrate derived from soy molasses supplied by Protein Technologies International (St. Louis, MO). Purity was determined to be >98% as analyzed by high-pressure liquid chromatography. Animals were killed on day 21 or weaned on that day for use at day 50 postpartum. This study was approved by the University of Alabama at Birmingham Animal Use Committee.

Immunohistochemistry. Uterine and vaginal tissues were removed rapidly, trimmed free of excess fat, fixed for 3 min in 100% methanol, and then placed in 4% paraformaldehyde overnight. Immunolocalization of TGF-α, EGF, and EGFR receptor was performed on trypsinized paraffin sections of uterine tissue by a modified avidin-biotin complex technique. A section of positive control tissue was run on each slide along with the test tissue, and a negative control slide with preimmune serum was analyzed with each batch of slides. Control tissues were skin for EGF receptor and kidney and/or skin for EGFR and TGF-α. To circumvent the problem of endogenous peroxidases, the tissue sections of 21- and 50-day-old animals were incubated with 3% aqueous hydrogen peroxide for 20 and 10 min, respectively. Primary antibodies were monoclonal mouse anti-TGF-α IgG, GF-10 (Oncogene Research Products, Cambridge, MA), polyclonal rabbit antirat EGF (Biomedical Technologies, Inc., Stoughton, MA), and rabbit polyclonal IgG raised against amino acid residues 1005–1016 of the human EGF receptor (Santa Cruz Biotechnol- ogy, Santa Cruz, CA). This EGF receptor antibody cross-reacts with rat tissue, and the amino acid sequence corresponds to the intracellular region of the receptor, precluding binding the soluble truncated form of EGF receptor found in rat (27). All primary antibodies were incubated overnight at 4°C in a humidity chamber with rocking (Orbitron Rotator II; Boekel, Scientific Products). TGF-α required a PBS/1%/BSA/1% milk solution for blocking. The secondary antibodies were biotinylated horse anti mouse IgG for TGF-α and goat antirabbit IgG (Vector Laboratories, Burlingame, CA) for EGF and EGFR receptor. Final rinses (three times) were performed with deionized water to preclude neutralizing the acetate buffer used in color development with 3-amino-9-ethylcarbazole tablets (Sigma Chemical Co., St. Louis, MO). Nuclei were counterstained with Gills No. 2 hematoxylin (Sigma) and tissue protected with Crystal Mount (Biomedia, Foster City, CA). After drying on a slide warmer (40–45°C) overnight,

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Immunohistochemical localization of TGF-α, EGF, and EGFR receptor in vagina of rats treated prepubertally with genistein and DMSO</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>TGF-α</td>
<td>EGF</td>
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<td>DMSO Genistein</td>
<td>DMSO Genistein</td>
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<td>Vagina from 21-day-old rats</td>
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<tr>
<td>Stroma</td>
<td>+       +       +       +       +       +       +       +       +       +       +       +       +</td>
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<tr>
<td>Epithelial layer</td>
<td>+       +       +       +       +       +       +       +       +       +       +       +       +</td>
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</tr>
<tr>
<td>Muscle tissue</td>
<td>+       +       +       +       +       +       +       +       +       +       +       +       +</td>
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</tr>
<tr>
<td>Blood vessel tunica</td>
<td>+       +       +       +       +       +       +       +       +       +       +       +       +</td>
<td></td>
</tr>
<tr>
<td>Blood plasma and cells</td>
<td>0       0       ++       ++       ++       ++       +       +       +       +       +       +       +</td>
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<td>Vagina from 50-day-old rats</td>
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<tr>
<td>Stroma</td>
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<td>Blood plasma and cells</td>
<td>+       +       +       +       +       +       +       +       +       +       +       +       +</td>
<td></td>
</tr>
</tbody>
</table>

* There was no EGF receptor immunostaining of plasma or blood cells noted in >90% of all the blood vessels; however, occasionally there were individual RBCs or clumps of RBCs that stained.
Permiston (Fisher Scientific, Pittsburgh, PA) was added, and the slides were coverslipped.

Immunohistochemical staining was performed in duplicate to verify results. Slides from six control and six treated animals were coded and analyzed blind on a Nikon light microscope with a Sony video camera and monitor. The overall staining pattern was evaluated at ×40–×100, with ×400 used to evaluate specific structures. Immunostaining was classified as “not detectable” (0), “detectable” (+), “moderate” (++) or “strong” (+++), or “intense” (++++) (Ref. 28).

Statistics. Data are expressed as mean ± SE. Differences were analyzed using Student’s t test (independent). Differences were considered statistically significant at P < 0.05.

References