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PHOSPHOTYROSINE INHIBITION AND CONTROL OF VASCULAR ENDOTHELIAL CELL PROLIFERATION BY GENISTEIN

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Abstract—Genistein (4′,5,7-trihydroxyisoflavone) is a potent anti-angiogenic compound. We investigated the inhibition of phosphotyrosine as a putative signaling mechanism utilized by the drug in modulating basic fibroblast growth factor (bFGF)-mediated vascular endothelial cell proliferation. The studies included the effect of genistein on DNA synthesis, cell viability, phosphotyrosine induction and characterization of the FGF receptor (FGFR). DNA synthesis was attenuated significantly by genistein in a concentration- and time course-dependent manner with relatively low cytotoxicity during a 16–24 hr exposure (IC_{50} = 12.5 μM; LC_{50} = 300 μM). Ligand-stimulated cells exhibited significant increases in phosphotyrosine, affecting FGFR and several tyrosine kinase substrates, ranging in size from M_r 28 to 200 kDa. Inhibition of phosphotyrosine induction as shown by western blots occurred only at high concentrations of the drug (>500 μM). These results were supported by results obtained using fluorescence immunocytochemistry. FGFR was shown to be FGF-R1β2, a dimer of approximately 85 and 62 kDa, which was prevented from being autophosphorylated when relatively high concentrations of the drug were applied. Low dose (<20 μM) inhibition of DNA synthesis by genistein did not correlate with the high concentration required for phosphotyrosine inhibition. The data suggest that although cell stimulation results in phosphotyrosine induction, inhibition of phosphotyrosine is not required for inhibition of DNA synthesis. Furthermore, in endothelial cells, inhibition of DNA synthesis by genistein is not mediated primarily by the inhibition of protein tyrosine kinase activity.

Key words: genistein; DNA synthesis; cell viability; phosphotyrosine; bFGF; protein tyrosine kinase; endothelial cells; FGF receptor

Protein tyrosine kinases are an important group of cellular enzymes associated with retroviruses and growth factor receptors. Tyrosine phosphorylation of proteins by PTKs† is crucial in the transduction of extracellular signals to the cell interior and the regulation of cell proliferation, differentiation and transformation. This provides an important premise for targeting PTK inhibition as a means of studying tyrosine phosphorylation mechanisms in the quest for therapeutic agents to control tumorigenic and angiogenic processes, both of which are affiliated with PTK activity (for reviews, see Refs. 1–4).

Some of the growth factors and agents known to possess angiogenic effects include acidic and basic fibroblast growth factor, transforming growth factor-alpha, insulin-like growth factor-1, platelet-derived growth factor, granulocyte macrophage and granulocyte colony stimulating factor, interleukin 6 [5–8], nitric oxide [7], vascular endothelial cell growth factor [9], and placenta growth factor [10]. FGFs comprise a family of several mitogenic polypeptides active in cells of mesenchymal, neuronal, and epithelial origin. bFGF occurs in the retina [11] and other parts of the body [12]. The FGFR gene encodes three protein tyrosine kinase gene products: *bek*, *flg* and *cek* [5] in multiple isoforms [13, 14]. The FGFs have been implicated in numerous vasoproliferative pathologies including those of ocular origin ([15]; for a review, see Ref. 16). The activity of bFGF is modulated by plasma membrane heparan sulfate proteoglycans, widely distributed in animal tissues (for detailed reviews, see Refs. 5, 17 and 18).

Although the mechanism of action of many naturally occurring protein tyrosine inhibitors is poorly understood, they offer considerable potential in the search for anti-oncogenic, anti-angiogenic agents (for reviews, see Refs. 1 and 19). Genistein, an isoflavone, isolated from soybean [20] and *Pseudomonas* [21], is reported to be a specific protein tyrosine kinase inhibitor of the EGF receptor [22, 23] and a potent anti-angiogenic compound [24]. Genistein inhibited kinase activity [25, 26] or phosphotyrosine [27–31] in some instances, but failed to achieve similar results in others [26, 32, 33]. However, because of recent interest in the anti-angiogenic properties of the drug, increased understanding of the biochemical pathway(s) should

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† Abbreviations: PTK, protein tyrosine kinase; FGF, fibroblast growth factor; bFGF, basic fibroblast growth factor; FGFR, fibroblast growth factor receptor; BAEC, bovine aortic endothelial cells; BCA, bicinchoninic acid; TBS, Tris-buffered saline; HRP, horseradish peroxidase; ECL enhanced chemiluminescence; and BrdU, bromodeoxyuridine.

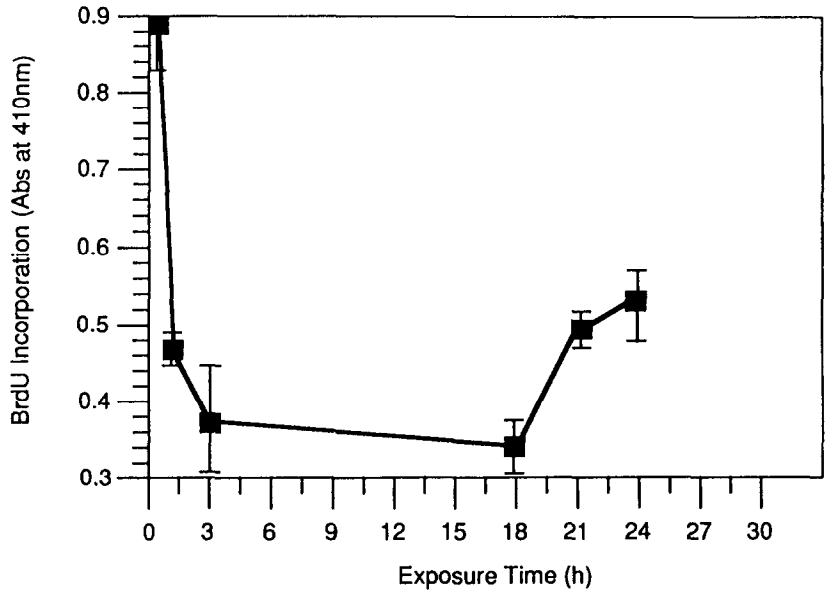

Fig. 1. Time course of the effect of genistein on BAEC proliferation. Approximately equal numbers of cells were seeded in 24-well plates (Falcon) to subconfluence (i.e. log phase). Prior to drug treatments, cells were made quiescent overnight in medium containing 1.5% calf serum. The medium was replaced with fresh medium containing 30 μM genistein, on a time course of 1, 2, 3, 18, 21, and 24 hr. Samples for each time course were replicated three times. Cells were stimulated with 100 ng/mL bFGF for 1 hr and then labeled with BrdU for 2 hr at 37°. The cells were then fixed in acetic acid/ethanol fixative, blocked in 3% BSA-Tween 20, and sequentially incubated in a monoclonal/nuclease mix, peroxidase anti-mouse IgG, and peroxidase substrate. The solution from each well was finally transferred to 96-well plates and absorbance per well measured at 410 nm, according to the protocol supplied in the BrdU cell proliferation assay kit (Amersham, U.K.). Values represent the mean ± SD of three replicates, corrected for background absorbance.

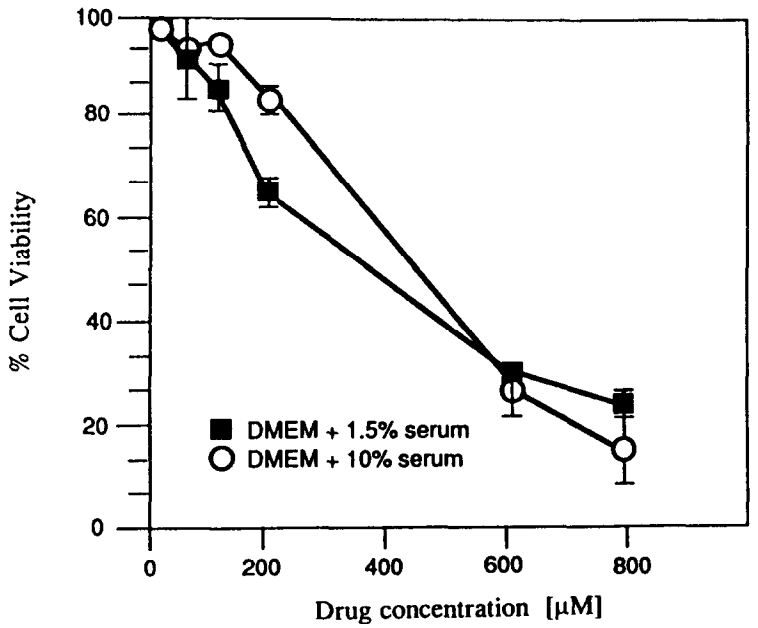

Fig. 2. Effect of genistein on the viability of BAEC. Cells cultured to confluence in 24-well plates (approximately 3×10^5 cells/well) were exposed to various concentrations of genistein and incubated overnight. Cells were then labeled with 0.33% neutral red dye for 2 hr at 37° and fixed by washing briefly in neutral red assay fixative. The dye was solubilized, and 100-μL aliquots were transferred to 96-well plates for spectrometric analysis at 540 nm in an ELISA plate reader, as described in Materials and Methods. Sensitivity to *in vitro* toxicity based on medium calf serum level was assessed by comparing cells cultured in 1.5% (■) with those cultured in 10% (○) calf serum-containing medium. The absorbance values reflect dye uptake and cell viability. Half-maximal viability of cells (i.e. LC_{50}) was approximately 300 μM. Data represent the mean with range of two replicates, corrected for background absorbance.

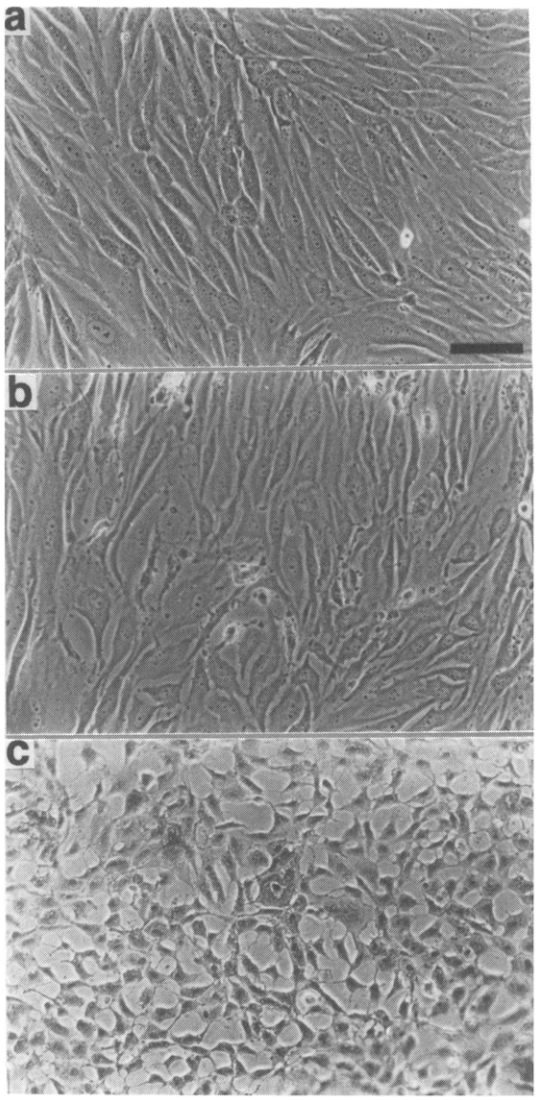

Fig. 3. Photomicrographs showing the effect of high concentration of genistein and duration of exposure on BAEC morphology. Cells incubated to confluence were either maintained in medium with no genistein (panel a—control) or exposed to a 600 μM concentration of the drug for 4 hr (panel b) or 8 hr (panel c). Cells were observed and photographed in the phase contrast mode using a Nikon Diaphot light microscope and camera. Bar = 100 μm.

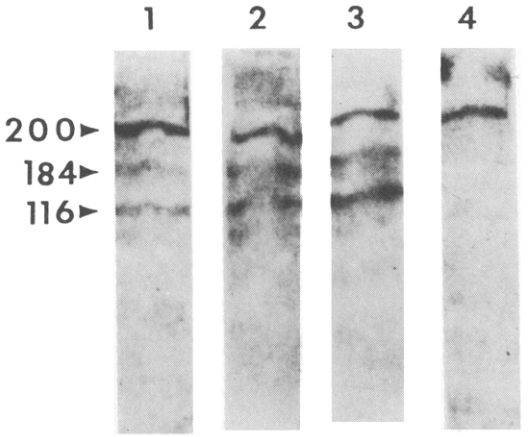

Fig. 4. Effect of genistein on bFGF-induced phosphotyrosine. Drug effects were compared with those of the analog diadzein and the solubilizing agent DMSO. Confluent cells quiescent overnight were pretreated for 2 hr in fresh medium containing 800 μM diadzein (lane 1), 5% DMSO (lane 2), 50 μM genistein (lane 3), or 800 μM genistein (lane 4). The monolayer surface was rinsed with PBS and then stimulated with fresh medium containing 100 ng/mL bFGF for 2 hr at 37°. Total proteins isolated from lysates were run on 4–20% polyacrylamide gradient minigel, electroblotted onto nitrocellulose membrane and then hybridized with anti-phosphotyrosine (UBI) antiserum, as detailed in Materials and Methods.

direct future research and promote effective therapeutic applications. This study addresses speculations that genistein exerts its antiproliferative action via the inhibition of protein tyrosine kinase activity [24, 26]. We determined that nullification of PTK activity is not the crucial step in the control of endothelial cell proliferation by this drug.

MATERIALS AND METHODS

Cell culture. Equal volumes of BAEC suspension were seeded in 6-well plates and incubated under standard conditions of 5% carbon dioxide at 37°. Culture medium comprised 10% fetal bovine serum, 90 μg/mL endothelial cell growth supplement and 1% antibiotic-antimycotic in Dulbecco's modified Eagle's medium (DMEM) (containing 1000 mg/L D-glucose, 25 mM HEPES buffer, 110 mg/L sodium pyruvate) (GIBCO, Grand Island, NY). The medium was changed at least once before a confluent monolayer of about 2×10^6 cells was attained.

Cell treatment with agonist (bFGF) and antagonist (genistein). Cells were made quiescent overnight in serum-free medium, exposed to various concentrations of drug, and then incubated for various times in medium containing 1.5% calf serum. After drug pretreatment, they were stimulated for 1 hr with 100 ng/mL bFGF at 37°. These procedures were modifications of Linassier *et al.* [26].

Protein isolation. Lysis buffer was added to cell monolayer (after discarding culture medium) and placed on ice for 20 min. The lysis buffer was made up of: 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, 35 μg/mL phenylmethylsulfonyl fluoride, 0.3 μg/mL EDTA, 0.7 μg/mL pepstatin A, 0.5 μg/mL leupeptin, 250 μM sodium orthovanadate and 50 μM sodium fluoride. Lysate was carefully harvested by scraping into 1.5 mL microfuge tubes. Cell debris was removed by centrifugation, and then equal volumes of 2× SDS-sample buffer (160 mM Tris–HCl, pH 6.8, 4% SDS, 30% glycerol, 5% β-mercaptoethanol, 10 mM

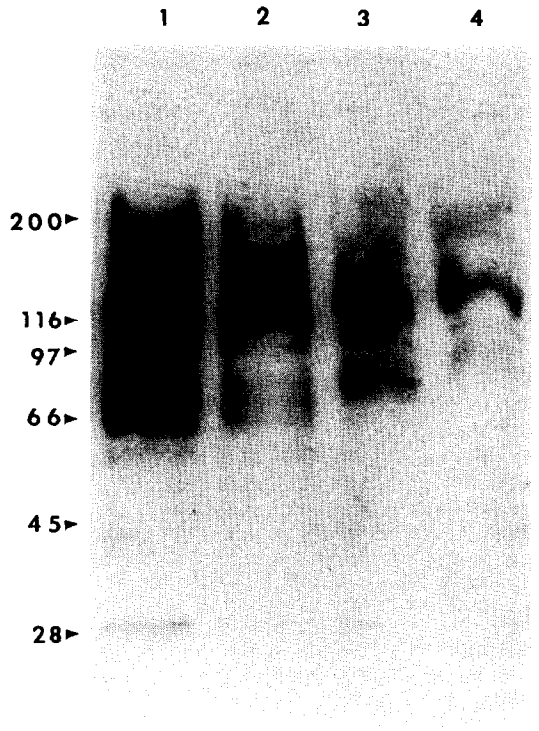

Fig. 5. Time course of phosphotyrosine inhibition by genistein at a 600 μM concentration. BAEC were cultured in 6-well plates in complete DMEM culture medium until confluent. Cells were made quiescent in serum-deprived medium overnight and then incubated at 37° in fresh medium containing 600 μM genistein for 2 hr (lane 1), 3 hr (lane 2), 4 hr (lane 3), or 5 hr (lane 4). The monolayer surface was rinsed with PBS and stimulated with bFGF (100 ng/mL) for 1 hr. Total protein was isolated from lysates, separated on 4–20% gradient SDS–polyacrylamide gel as described in Materials and Methods. Proteins were electroblotted onto Immobilon-P (Millipore) membranes using the trans-Blot SD (BIO-RAD) apparatus. Tyrosine phosphorylated proteins were detected on Kodak X-ray films by ECL (Amersham, U.K.).

dithiothreitol, 0.01% bromophenol blue) were added to the lysate and boiled for 5 min at 100°. Sample preparation was performed according to modifications of Wong *et al.* [34]. Protein concentration was determined by the Pierce BCA® assay technique, and samples were stored at −70°, until ready for electrophoretic analysis.

Immunoprecipitation. Anti-bFGF receptor antibody (UBI, Lake Placid, NY) was added to lysate, vortexed and incubated on ice for 2 hr. This was followed by the addition of protein A-agarose to obtain an antigen–antibody–agarose complex. This complex was incubated on ice for 2 hr followed by elution of FGFR by serial washing in 100 μL PBS, PBS–0.5 M NaCl and lysis buffer. Each wash step involved pelletization and resuspension for 5 min at 1000 *g*. The immunoprecipitate was finally resuspended and boiled for 5 min in 100 μL SDS-sample buffer.

SDS–PAGE. Equal amounts of protein samples were run at a constant 200 V for 45 min on 4–20%, 0.75 mm thick, precast, polyacrylamide minigels (BIO-RAD Laboratories, Richmond, CA), using a miniprotean apparatus (BIO-RAD). Broad range, biotinylated (6.5 to 200 kDa) and prestained (18.5 to 106 kDa) molecular weight protein markers (BIO-RAD) were run simultaneously with the samples. Electrophoresis was performed according to Laemmli [35].

Electroblotting, hybridization and autoradiography. Gels were equilibrated in Towbin's transfer buffer and then electroblotted on 0.4 μm Immobilon-P membranes (Millipore, Bedford, MA) using the trans-Blot SD (BIO-RAD) apparatus. Membranes were blocked in 5% nonfat dried milk for 1 hr and probed with antiphosphotyrosine antibody (UBI) at a 1:1000 dilution in TBS, for a minimum of 2 hr at room temperature, or 16 hr at 4°. Blots were washed in TBS and 3% Tween 20 and incubated for 1 hr at room temperature in a double secondary antibody matrix composed of IgG-HRP for detecting protein samples (diluted 1:1000) and streptavidin-HRP (for detection of biotinylated molecular weight markers), diluted 1:20,000 in TBS containing 3% BSA. When necessary, signals were enhanced by first incubating membranes in biotinylated-IgG secondary antibody (at 1:20,000), washed in TBS, and then incubated in HRP-conjugated secondary antibody. Blots were washed (three times, 5 min each), immersed in ECL immunodetection reagents, and then exposed to Kodak X-ray film from 15 sec to 5 min, as described in Amersham ECL protocols.

Coomassie Blue staining of total proteins. SDS–polyacrylamide gels were fixed in 45% methanol and 10% acetic acid aqueous solution for 10 min, soaked in saturated picric acid solution for increased sensitivity, and then stained in 0.25% aqueous Coomassie Brilliant Blue (R250), for a minimum of 2 hr. Gels were destained in several washes of 10% acetic acid solution.

DNA synthesis. DNA synthesis was measured using tritiated thymidine ([^{3}H]TdR) and BrdU incorporation assays. A BrdU cell proliferation kit was supplied by Amersham (U.K.). BrdU assay, like tritiated thymidine, is based on the incorporation of label by replicating DNA. Equal volumes of cell suspension (~2×10^4) were seeded in 24-well tissue culture plates (Falcon) and cultured to subconfluence under standard conditions. Prior to treatments, cells were made quiescent overnight in serum-free medium and treated with different concentrations of genistein in DMEM containing 1.5% serum for 24 hr. To study the effects of duration of exposure, 30 μM genistein was administered to a subconfluent monolayer on a time course. Each time period was replicated three times. Growth stimulation and labeling was carried out simultaneously by replacing test medium with labeling medium (100 μCi/mL [^{3}H]TdR or BrdU) and agonist (100 ng/mL bFGF) for 2 hr. Samples were incubated at 37° for 3–4 hr. Cells were solubilized, and label incorporation was assayed by spectrometry (ELISA plate reader at 410 nm absorbance for BrdU, liquid scintillation counter for tritiated thymidine incorporation) according to Livneh *et al.* [36].

Cell viability. Cells cultured in 24-well tissue

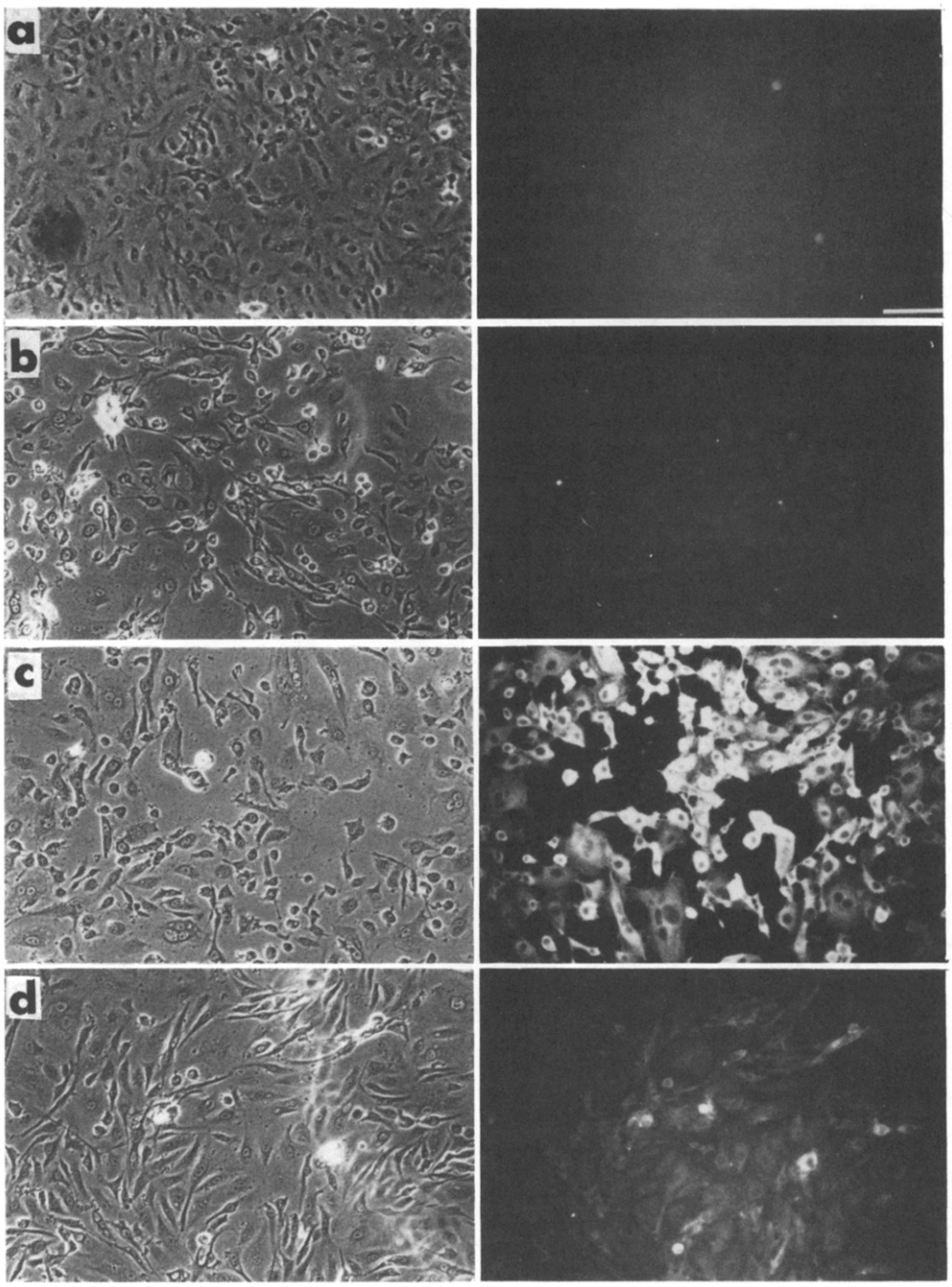

Fig. 6. Photomicrographs showing the effect of genistein on the induction and cytolocalization of bFGF-induced phosphotyrosine detected by immunofluorescence in BAEC. Equal numbers of cells were seeded in Permanox 4-well chamber slides (Nunc) until confluent. The cells were then made quiescent in medium containing 1.5% calf serum overnight. Cells were either treated with 600 μM genistein for 4 hr at 37° (panel d) or maintained in the same culture medium (panels a, b, and c). The cells were subsequently stimulated with bFGF (100 ng/mL) for 1 hr (panels a, c, and d) or maintained in the same culture medium—unstimulated control (panel b). The culture medium was discarded, and the cell monolayer was rinsed twice with PBS/Tween 20 followed by fixation in ethanol–acetone (1:1) for 30 min, at room temperature. The cells were blocked in normal non-Ig serum for 1 hr at room temperature. The blocking solution was replaced by a solution of anti-phosphotyrosine antiserum (1:100) (UBI) containing 1 mM sodium orthovanadate (panels b, c, and d), or normal serum–control (panel a) and incubated for 1 hr at room temperature after performing an initial serial dilution to optimize the concentration of the primary antibody. Cells were again washed in PBS/Tween 20 and incubated in biotinylated anti-mouse IgG (1:100) for 1 hr at room temperature. The cells were washed in PBS/Tween 20 and incubated in fluorescein–streptavidin (1:50) prior to mounting in fluoromount G (FisherBiotech, Pittsburgh, PA). The chamber partitioning-plastic was replaced with a coverslip, and the slides were observed for photomicrography in phase contrast and fluorescent modes using a ZEISS Axioskop microscope and camera. Phosphotyrosine inhibition patterns agree with the outcome of the western immunodetection assay. Notice the dark (phosphotyrosine-void) central position of the nucleus of stimulated cells (panel c, immunofluorescence). Bar = 100 μm.

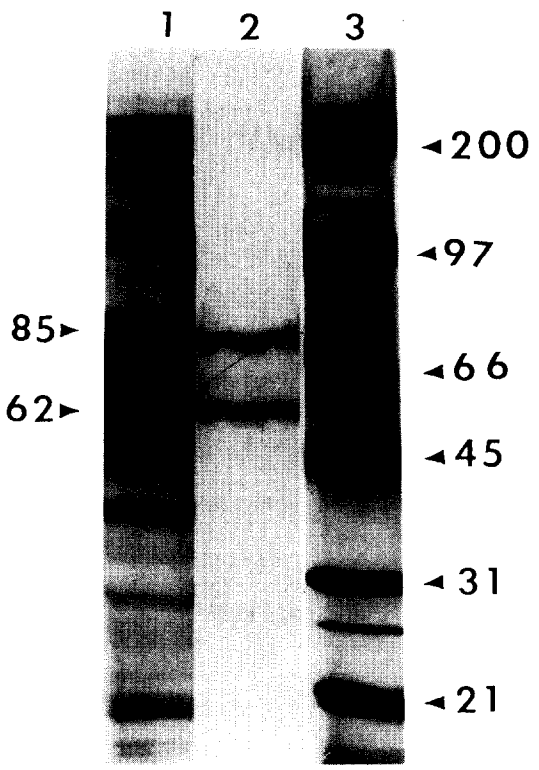

Fig. 7. Coomassie-stained FGFR immunoprecipitate and total cell lysate. Cell lysate was immunoprecipitated with polyclonal antibody to FGFR (UBI, Cat. No. 06-177). Details of this procedure are described in Materials and Methods. Samples were run on a 4–20% gradient SDS–polyacrylamide minigel and stained with Coomassie-Brilliant Blue. The location and size of the immunoprecipitated FGFR subunits (lane 2) were confirmed by comparing with total cell lysates (lane 1) and molecular weight standards (lane 3). The broad range (6.5 to 200 kDa) molecular weight markers were procured from BIO-RAD.

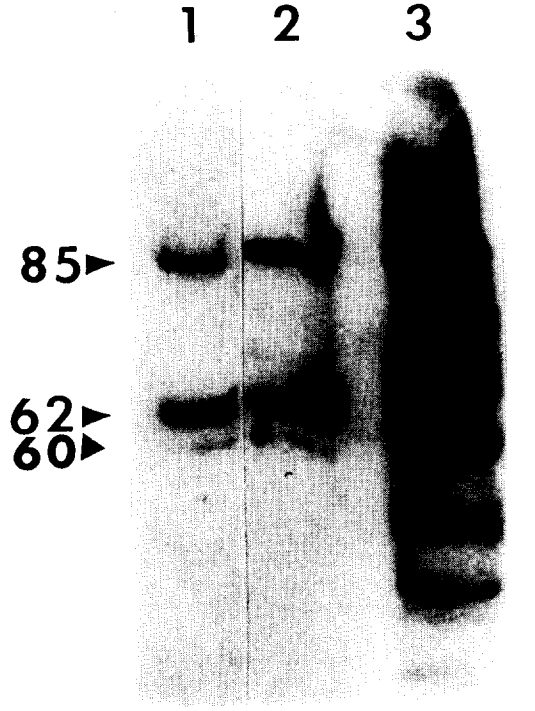

Fig. 8. Identification of FGFR using western immunoblot. Lysates derived from bovine aortic (lane 1) and human retinal capillary (lane 2) endothelial cells were subjected to SDS–PAGE on a 4–20% gradient gel, electroblotted onto PVDF membrane (Millipore) and processed as described in Materials and Methods. The anti-FGFR antiserum (UBI, Cat. No. 06-177) is an external region peptide corresponding to the deduced cDNA sequence of the chicken FGFR. The positive control for the FGFR (UBI, Cat. No. 12-001) (lane 3) confirms the identity of the FGFR bands.

culture plates were used for *in vitro* toxicological studies to determine the effects of exposure to different concentrations of genistein on cell viability. The neutral red assay (Sigma, St. Louis, MO), originally developed by Borenfreund and Puerner [37], is based on the active uptake of the neutral red dye into the lysosomes of living cells only. Cells were incubated in either complete culture medium (containing 10% calf serum) or medium containing only 1.5% serum, overnight. Filter-sterilized neutral red dye (0.33%) was added in a volume equal to 10% of the culture medium per well and incubated for 4 hr. The medium was removed and the cells were fixed by washing briskly in neutral red assay fixative (0.1% calcium chloride in 0.5% formaldehyde). Solubilization solution (1% acetic acid in 50% ethanol) equal to the original volume of culture medium was added, and the plate left to stand for 10 min at room temperature. The solution in each well was then transferred to 96-well plates, and absorbance was measured spectrophotometrically at 540 nm, using an ELISA plate reader.

Immunofluorescence cytochemistry. Equal volumes of cell suspension were incubated in four-chamber Permanox® slides (Nunc, Naperville, IL) and incubated to confluence. Cells were made quiescent by replacing culture medium with serum-free medium, overnight. Each chamber received a different treatment based on the test objective. Each test was replicated three times. Controls either received no bFGF stimulation or were probed with normal non-IgG serum instead of antiserum. Cells were treated for fluorescence immunodetection according to Johnson and Thorpe [38]. Fluorescence microscopy and photography were performed using the ZEISS® Axioskop microscope and camera.

RESULTS

Proliferative response. Studies carried out using both BrdU and tritiated thymidine were mutually supportive. Absorbance values showed that the inhibitory effect of genistein on DNA synthesis was concentration dependent with $IC_{50} = 12.5\ \mu M$ (results not shown). It was also shown that at a low concentration of 30 μM, the effect was time course dependent, the suppressive effect abating with time,

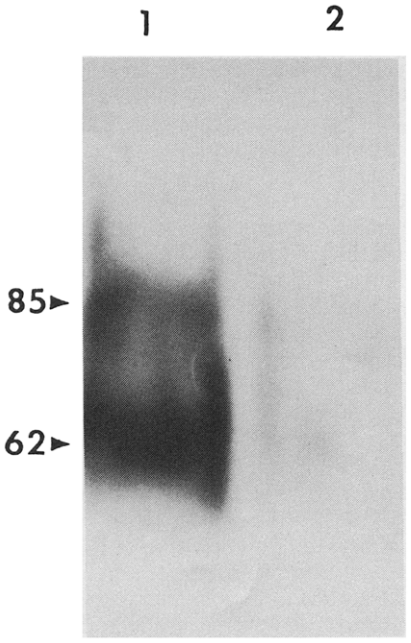

Fig. 9. Inhibition of bFGF receptor autophosphorylation by genistein. Confluent cells were serum-starved overnight and either directly stimulated with bFGF (100 ng/mL) for 1 hr (lane 1) or treated with 800 μM genistein for 4 hr, prior to stimulation with bFGF (lane 2). FGFR immunoprecipitate was run on 4–20% gradient SDS–polyacrylamide minigel, electroblotted and hybridized with anti-phosphotyrosine (UBI). Immunoprecipitation, western transfer, and immunodetection of phosphotyrosine were performed as described in Materials and Methods.

allowing DNA synthesis to resume after 18 hr (Fig. 1).

Cell viability. In vitro toxicology assays using neutral red dye uptake indicated that between 16 and 24 hr cell viability was relatively high. The high percentage of viable cells at relatively high concentrations resulted in a high half-maximal viability concentration (i.e LC_{50} = 300 μM) (Fig. 2). Cells incubated in 10% serum showed a slightly higher percentage of viability than those incubated in 1.5% serum. A deleterious effect on cell morphology was observed only at concentrations greater than 600 μM, manifested by loss of the typical spindle or cobblestone layout and the appearance of intercellular voids, apparently due to loss of adhesion to adjacent cells (Fig. 3).

Effect of genistein on phosphotyrosine inhibition. Endothelial cells stimulated by bFGF, unlike quiescent cells, showed an increase in number and intensity of tyrosine phosphorylated substrates, affecting tyrosine phosphoproteins ranging in size from 200 to 28 kDa. At low concentrations (20–120 μM) of genistein, there was no detectable inhibition of tyrosine phosphorylation of these substrates (results not shown). However, at 800 μM pretreatment, ligand-stimulated cells showed marked inhibition of phosphotyrosine compared with those treated with the genistein analog diadzein, and the solubilizing agent DMSO (Fig. 4). Genistein inhibited all but a single band of 200 kDa. To determine whether the inhibitory effect of the drug was related to duration of exposure, cells were incubated in 100 and 600 μM on a time course. It was shown that phosphotyrosine inhibition occurred only slightly at 100 μM, as indicated by loss of signal in several PTK substrates (results not shown). However, the effect of time was more intense at a higher concentration of 600 μM (Fig. 5) with the disappearance of signal in the often prominent 28 kDa substrate protein preceding the others. When

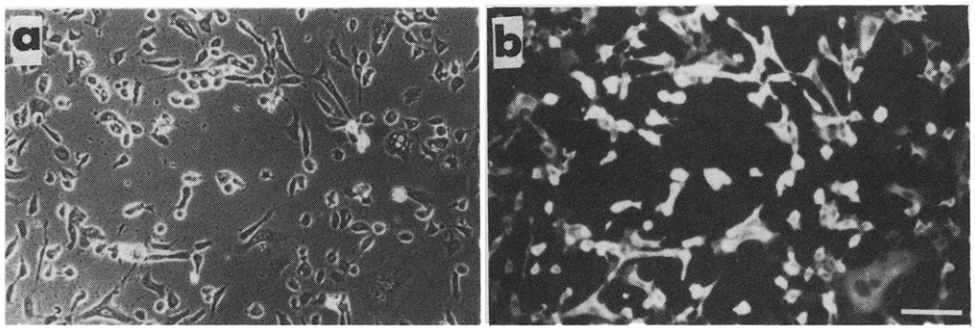

Fig. 10. Photomicrographs showing cytolocalization of bFGF receptor detected by immunofluorescence in BAEC. Cells were cultured to confluence in Permanox multiwell chamber slides (Nunc), fixed in ethanol–acetone (1:1), and then blocked with normal serum for 1 hr. The blocking solution was replaced by a solution of anti-bFGF antiserum (1:100) and incubated for 1 hr at room temperature, after performing an initial serial dilution to optimize the concentration of the primary antibody. Cells were washed in PBS/Tween 20 and incubated in biotinylated anti-mouse IgG (1:100) for 1 hr at room temperature. The cells were then washed in PBS/Tween 20 and incubated in fluorescein–streptavidin (1:50) before mounting in fluoromount G (FisherBiotech). The chamber-partitioning plastic was replaced with a coverslip, and the slides were observed for photomicrography in phase contrast (a) and fluorescent (b) modes, using a ZEISS Axioskop microscope and camera. Bar = 100 μm.

the effect of the drug was examined in whole cells by fluorescence cytochemistry, it was found that phosphotyrosine levels were increased markedly in FGF-stimulated cells, localized mainly outside the nucleus (Fig. 6c). At high concentrations of genistein lasting 4 hr, it was shown that levels of phosphotyrosine were suppressed markedly (Fig. 6d). These findings support earlier results obtained from western immunoblots.

Characterization of FGFR. When FGFR was immunoprecipitated using polyclonal antiserum and stained with Coomassie Blue, two bands of approximately 85 and 62 kDa were detected. These bands were also observed in total cell lysates (Fig. 7). A third band (<62 kDa) was detected in immunoblots of total cell lysates but not in immunoprecipitated FGFR (Fig. 8). The FGFR immunoprecipitate from stimulated cells showed strong phosphotyrosine signals that were lost when the cells were exposed to a high concentration of 0.8 mM (Fig. 9). In untreated whole cells, FGFR was shown to be distributed over entire cell surfaces (Fig. 10).

DISCUSSION

The activity of protein tyrosine kinase is often associated with mitogenesis in endothelial cells. Our results on the effect of genistein on the inhibition of DNA synthesis are consistent with earlier studies [24, 26, 39]. The results show that not only is the inhibitory action of the drug on DNA synthesis concentration dependent, but it is also time course dependent. The relatively high efficacy in inhibiting cell proliferation ($IC_{50} = 12.5\ \mu M$) coupled with a low cytotoxicity ($LC_{50} = 300\ \mu M$) suggest that the drug utilizes an efficient growth modulation mechanism that suppresses cell proliferation with minimal injury and cell death. It is this property that probably accounts for the high tolerance of this drug in humans [24]. Dissipation of its inhibitory effects may also suggest that the drug–receptor binding is reversible and less likely to impose permanent disruption of normal cell functions. Cell viability is adversely affected only at high concentrations (>600 μM), resulting in impairment of cell morphology. This suggests a definite limit of cell tolerance to drug concentration.

It is not clear what mechanism of action is utilized by genistein in exerting its anti-proliferative activity in cells because the existing reported data are conflicting [15, 22, 33]. In this study, we show that the inhibition of phosphotyrosine in FGF-stimulated cells did not occur at similar low concentrations where the drug effectively inhibited DNA synthesis. Since phosphotyrosine induction is mediated by protein tyrosine kinase, this outcome does not conform to the expectation that the inhibition of DNA synthesis (and therefore cell proliferation), is mediated primarily by the inhibition of protein tyrosine kinase. These findings agree with those obtained in rat adipocytes [33], human prostate cancer cells [40], and others [32, 41] in which the drug manifested a very weak effect on protein phosphorylation even at high concentrations. Such results point to non-tyrosine kinase specificity of the drug and may explain in part why it inhibits DNA synthesis in Chang liver cells whether stimulated by growth factor or not [23]. The increased level of phosphotyrosine in bFGF-stimulated cells shown in this study provides indirect evidence that phosphotyrosine induction is an integral part of endothelial cell mitogenesis. However, the continuous presence of phosphotyrosine in growth-arrested conditions imposed by the drug suggests that the signaling mechanisms employed by the drug in controlling cell growth are not directly coupled to protein tyrosine kinase activity. Thus, mitogenesis is blocked via a process(es) that bypasses tyrosine kinase receptor action.

We show that bFGF-induced phosphotyrosine was localized mainly outside the nucleus. This distribution pattern correlates with the general intracellular sequestration of protein tyrosine kinases in the cell, allowing access to different sets of PTK substrates [2, 42]. The FGF receptor was shown to be distributed in a diffuse manner over the entire cell surface. This pattern is consistent with earlier reports [43] and seems to be critical to its role as a signal transducer working inwards from the extracellular domain. The FGF receptor is known to exhibit a cell-specific expression of the *flg* gene and isoforms of it that result from splice variations [14]. In this study, the FGFR immunoprecipitate was shown to be a dimer containing subunits of approximately 85 and 62 kDa, which correspond to those of FGF-R1β2(~85 and 60 kDa), an isoform of the *flg* receptor reported by Xu *et al.* [14]. The dimeric isoform was described as having two β immunoreactive epitopes in the extracellular domain, an ATP binding site, and a kinase catalytic domain [14, 43]. A third band of approximately 60 kDa observed in western blots may be a product of proteolytic degradation since it was not observed in immunoprecipitates. Ligand-stimulation resulted in autophosphorylation of FGFR. This autophosphorylation was absent when higher concentrations of the drug were applied, consistent with the fact that low concentrations of the drug were not sufficient to inhibit tyrosine kinase activity and phosphotyrosine induction, and demonstrates further that protein tyrosine kinase receptor may only have an insignificant role since action by the drug in preventing autophosphorylation occurs only at very high concentration, and not at the low concentration range where inhibition of DNA synthesis was evident. The non-toxic suppression of the immune response of T cells (affording potential therapy in allograft rejection) [44], suppression of cancer cell growth [40], and inhibition of angiogenesis [24] by genistein are probably a function of multiple signal transducing mechanisms. While it seems largely true that the drug is an efficient antiproliferative agent in different cell types, its mode of action may not be universal. In particular, its potency as a protein tyrosine kinase-specific inhibitor should be treated on the merits of the experimental conditions. Although the effect of genistein on PTKs may be secondary in the attenuation of DNA synthesis in vascular endothelial cells, this does not necessarily detract from the non-toxic antiproliferative capacity of the drug. We have shown in this study that the drug markedly inhibits

protein tyrosine kinase in vascular endothelial cells but at considerably higher concentration than expected. Therefore, it seems that the mechanism of low dose inhibition involves an alternate pathway(s) not tightly coupled to tyrosine kinase activity.

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