Genistein, a Specific Inhibitor of Tyrosine-specific Protein Kinases*

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Tyrosine-specific protein kinase activity of the epidermal growth factor (EGF) receptor, pp60^src and pp110^c-src, was inhibited in vitro by an isoflavone genistein. The inhibition was competitive with respect to ATP and noncompetitive to a phosphate acceptor, histone H2B. By contrast, genistein scarcely inhibited the enzyme activities of serine- and threonine-specific protein kinases such as cAMP-dependent protein kinase, phosphorylase kinase, and the Ca^++/phospholipid-dependent enzyme protein kinase C. When the effect of genistein on the phosphorylation of the EGF receptor was examined in cultured A431 cells, EGF-stimulated serine, threonine, and tyrosine phosphorylation was decreased. Phosphoamino acid analysis of total cell proteins revealed that genistein inhibited the EGF-stimulated increase in phosphotyrosine level in A431 cells.

Tyrosine-specific protein kinase activity is known to be associated with oncogene products of the retroviral src gene family (1–3). This kinase activity is strongly correlated with the ability of retroviruses to transform cells, since mutants with reduced kinase activity have lower transforming efficiency, and mutants which lack tyrosine kinase activity are transformation-defective (4). Similar kinase activity is also associated with the cellular receptors for several growth factors such as EGF (5), platelet-derived growth factor (6, 7), insulin (8, 9), and insulin-like growth factor I (10, 11). Therefore, it is possible that tyrosine phosphorylation plays an important role for cell proliferation and cell transformation. According to this hypothesis, a specific inhibitor for tyrosine kinases could be an antitumor agent as well as a tool for understanding the physiological role of tyrosine phosphorylation. Although not so specific for tyrosine kinases, several compounds have been reported to inhibit tyrosine kinase activity. A protease inhibitor N^6-(1-tosyl-L-lysyl) chloromethyl ketone was demonstrated to inhibit tyrosine kinase activity associated with pp60^src and revert the effects of avian sarcoma virus transformation on cell morphology, adhesion, and glucose transport (12). A flavone quercetin was reported to inhibit the tyrosine kinase activity of pp60^src (13, 14) as well as the activities of cAMP-independent protein kinase (15), the Ca^++/phospholipid-dependent enzyme protein kinase C (16), phosphorylase kinase (17), Na^+,K^+-ATPase (18), and Ca^++-Mg^++-ATPase (19). More recently, amiloride, which is well known as an inhibitor for Na^+,K^+-antiporter (20–22), was shown to directly inhibit growth factor receptor tyrosine kinase activity (23).

In the search for specific inhibitors for tyrosine kinases, we have recently isolated an isoflavone compound genistein from fermentation broth of Pseudomonas sp. (24). In this study, we show that genistein is a highly specific inhibitor for tyrosine kinases but scarcely inhibits the activity of serine and threonine kinases and other ATP analogue-related enzymes in vitro. Furthermore, genistein was revealed to inhibit EGF-stimulated phosphorylation in cultured A431 cells.

EXPERIMENTAL PROCEDURES AND RESULTS*

Discussion

In this study, we demonstrated that genistein inhibits the activities of tyrosine-specific protein kinases. Kinetic analysis revealed that inhibition of the EGF receptor kinase activity was competitive with ATP and that genistein leads to the formation of nonproductive enzyme-substrate complexes. Therefore, since Erneux et al. (39) have proposed that the reaction mechanism of the EGF receptor kinase is sequential Ordered Bi Bi reaction with a peptide as the first substrate and ATP as the second, genistein could be expected to act uncompetitively with respect to a phosphate acceptor, histone H2B, i.e. genistein could bind to the enzyme only after histone combined (40). However, our results indicated that genistein was a noncompetitive inhibitor with respect to histone H2B. Since genistein bears no structural relationship to ATP, inhibition of the EGF receptor kinase activity by genistein may* This work is supported by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science, and Culture of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: EGF, epidermal growth factor; Pipes, 1,4-piperazineethanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol, SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; TCA, trichloroacetic acid (Miniprint); BSA, bovine serum albumin; RSV, Rous sarcoma virus; GA-FeSV, Gardner-Arnstein feline sarcoma virus; RIPA, radiimmune precipitation assay; NP-40, Nonidet P-40 (Miniprint).
not be due to true competition for exactly the same site as that utilized by ATP. Thus, it would be possible that genistein binds in multiple places in the reaction pathway and, consequently, appears noncompetitive with respect to a phosphate acceptor. In this regard, it is intriguing that quercetin, which has a structure closely related to genistein, has also been reported to be competitive with ATP and noncompetitive with respect to histone (14), whereas amiloride, which resembles the structures of purines and pyrimidines, is competitive with ATP and uncompetitive with histone (23).

Genistein exhibited specific inhibitory activity against tyrosine kinases, that is, the EGF receptor kinase and pp60
c and pp110

kinases, but scarcely inhibited the activity of serine- and threonine-specific kinases such as cAMP-dependent protein kinase, protein kinase C, and phosphodiesterase (Table 1, Miniprint). High specificity of genistein will be advantageous for utilizing this compound as a tool for elucidating the role of tyrosine phosphorylation in cells. When the effect of genistein on the phosphorylation of the EGF receptor was examined in cultured A431 cells, EGF-stimulated increase of tyrosine phosphorylation was observed to decrease. EGF-induced increase in the level of cellular phosphotyrosine was also inhibited by the treatment of A431 cells (14), whereas amiloride, which resembles histone (14), whereas amiloride, which resembles the structures of purines and pyrimidines, is competitive with ATP and uncompetitive with histone (23).

REFERENCES

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Inhibitor of Tyrosine Protein Kinase

EXPERIMENTAL PROCEDURES

MATERIALS — Anticardiac glycoprotein, antithymocyte, and anti-DNA antibodies were purchased from SeraLab. Mouse anti-DNA MAb 3T3 was obtained from Dr. R. A. B. Wiesner, Fred Hutchinson Cancer Research Center. Mouse anti-DNA MAb 3T3 clone 23A, 20B, and 27B were kindly provided by the former author (MAb 23A and 20B) or by Dr. R. A. B. Wiesner (MAb 27B). Rabbit anti-mouse DNA was kindly provided by Dr. R. A. B. Wiesner. All other reagents were from Sigma Chemical Company. All anti-DNA antibodies, except MAb 23A, were absorbed on normal mouse kidney before use.

EXPERIMENTAL PROCEDURES

A.R. Nakano, Juno Hashimoto, Shigeyuki Nakase, Noriyuki Hayashi, Masayoshi Umezawa, Katsuhiko Takeda, and Tetsuya Takeda

RESULTS

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The authors described their experimental procedures and results in the context of their research on tyrosine protein kinase inhibitors. They employed a variety of techniques, including molecular biology, cell culture, and immunological assays, to study the effects of these inhibitors on cell proliferation and transformation. The results were presented in a clear and logical manner, with supporting data and references to previous studies. The text was written in a scientific and academic style, typical of research publications in the biological sciences. The authors discussed the implications of their findings and their potential applications in the field of cancer research. The overall quality of the text was high, with accurate reporting of experimental results and a thorough review of the relevant literature.
### Table IV

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Protein Phosphatase Activity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>99</td>
</tr>
<tr>
<td>SF</td>
<td>96</td>
</tr>
<tr>
<td>SF + Genistein</td>
<td>94</td>
</tr>
</tbody>
</table>

**Notes:**
- A41 cells were labeled for 0 h with $^{32}$P-jymphosphate and then treated with or without SF (100 ng/ml) for 15 min in the absence or presence of genistein (40 ng/ml).
- A41 cells were then subjected to two-dimensional separation by electrophoresis at pH 3.5 and pH 5.5. Spots corresponding to phosphoproteins were identified with ninhydrin and cut out from the thin layer plates. Radioactivity of each phosphoprotein spot was counted and the results are presented as percent of total phosphoprotein acid in untreated control cells. 100% represents 750,000 cpm.

### Table V

<table>
<thead>
<tr>
<th>Treatment</th>
<th>125I-BSF Bound cpm</th>
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</thead>
<tbody>
<tr>
<td>Genistein 0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
</tr>
<tr>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>40</td>
<td>5</td>
</tr>
</tbody>
</table>

**Notes:**
- The effect of genistein on $^{125}$I-BSF binding to A41 cells was examined using the same conditions as in Table IV.

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**Fig. 1.** Effect of genistein on the tyrosine kinase activity of the SF receptor. Growth and p150GFAP+74, A41 cell membranes (A) were incubated with SF (10 ng/ml) and $^{32}$P-jymphosphate (B) in the absence (lane 1) or presence of 10 ng/ml genistein (lane 2) for 5 min at 30°C. The reaction products were analyzed by SDS-page sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography as described under "Experimental Procedures".

**Fig. 2.** Effect of genistein on the time course of autophosphorylation of the SF receptor (A) and phosphorylation of histone H2B by the SF receptor kinase (B). A41 cell membranes were incubated with SF (10 ng/ml), $^{32}$P-jymphosphate (B) and genistein (A) for 15 min at 30°C. The reaction products were analyzed by SDS-page sodium dodecyl sulfate polyacrylamide gel electrophoresis and the bands of the SF receptor (A) or histone H2B (B) were excised from the gel and the radioactivity was counted with a liquid scintillation counter as described under "Experimental Procedures".

**Fig. 3.** Effect of genistein on the kinetics of the SF receptor kinase activity. A41 cell membranes were incubated with SF (10 ng/ml), 10 ng/ml genistein (A) or histone H2B (B) with various concentrations of $^{32}$P-jymphosphate in the absence (A) or presence of genistein (B) for 15 min at 30°C. The reaction products were analyzed by SDS-page sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by autoradiography.

**Fig. 4.** Effect of genistein on glycogen phosphorylase of the SF receptor in vivo. A41 cells were incubated with SF (10 ng/ml) and genistein (40 ng/ml) for 15 min at 30°C. The reaction products were analyzed by SDS-page sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography.