Intratumoral Localization of Aromatase and Interaction between Stromal and Parenchymal Cells in the Non–Small Cell Lung Carcinoma Microenvironment

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Abstract

Estrogens produced as a result of intratumoral aromatization has been recently shown to play important roles in proliferation of human non–small cell lung carcinomas (NSCLC), but the details have remained largely unknown. Therefore, in this study, we evaluated the possible roles of intratumoral aromatase in NSCLCs as follows: (a) evaluation of intratumoral localization of aromatase mRNA/protein in six lung adenocarcinoma cases using laser capture microdissection combined with quantitative reverse transcriptase-PCR and immunohistochemistry; (b) examination of the possible effects of isolated stromal cells from lung carcinoma tissues on aromatase mRNA transcript expression in lung carcinoma cell lines (A549 and LK87) through a coculture system; and (c) screening of cytokines derived from stromal LK001S and LK002S cells using cytokine antibody arrays and subsequent evaluation of effects of these cytokines on aromatase expression in A549 and LK87. Both aromatase mRNA and protein were mainly detected in intratumoral carcinoma cells but not in stromal cells. Aromatase expression of A549 and LK87 was upregulated in the presence of LK001S or LK002S cells. Several cytokines such as interleukin-6 (IL-6), oncostatin M, and tumor necrosis factor-α, all known as inducible factors of aromatase gene, were detected in conditioned media of LK001S and LK002S cells. Treatment of both oncostatin M and IL-6 induced aromatase gene expression in A549 and LK87, respectively. These results all indicated that intratumoral microenvironments, especially carcinoma-stromal cell interactions, play a pivotal role in the regulation of intratumoral estrogen synthesis through aromatase expression in human lung adenocarcinomas. Cancer Res 70(16): 6659–69. ©2010 AACR.

Introduction

Non–small cell lung carcinomas (NSCLC) account for ~80% of all lung carcinomas and are composed of heterogeneous groups such as adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma. Squamous cell carcinomas comprise 57% of all lung cancer in men and 25% in women, whereas adenocarcinomas comprise 30% cases in men and 55% in women (1). Therefore, sex steroid hormones, such as estrogen, may also play an important role in NSCLC, especially adenocarcinoma. In postmenopausal breast carcinoma patients, intratumoral production of estrogens occurs as a result of aromatization of androgens into estrogens and is catalyzed by the cytochrome P450 aromatase enzyme (2, 3). In 2005, Weinberg and colleagues showed aromatase-dependent cell proliferation of lung carcinoma using a mice xenograft model (4). Since the pioneering study of Weinberg and colleagues (4), aromatase has been studied as one of the therapeutic targets for lung carcinoma patients (5–7). Mah and colleagues (5) reported an association between aromatase expression and adverse clinical outcome in female patients with early-stage NSCLC. These findings all suggest that intratumoral aromatase plays pivotal roles in estrogen-dependent pathways in NSCLC as well as in breast carcinoma. Phase II clinical trials of a combination therapy using an epidermal growth factor receptor inhibitor [erlotinib (Tarceva)] and an estrogen receptor (ER) blocker (fulvestrant) versus erlotinib alone in NSCLC patients are now under way (ClinicalTrials.gov identifier: NCT00100854 and NCT00592007). In addition, a phase II randomized trial of fulvestrant and an aromatase inhibitor (anastrozole) as consolidation therapy in postmenopausal...
women with advanced NSCLC who have received first-line platinum-based chemotherapy with or without bevacizumab will also be scheduled (ClinicalTrials.gov identifier: NCT00932152). However, the basic biological characteristics, such as tissue localization and regulation of aromatase expression in the lung carcinoma microenvironment, are generally considered prerequisites for successful outcome of these novel therapies but have remained relatively unknown.

Therefore, in this study, we first examined the localization of aromatase in six lung adenocarcinoma and three squamous cell carcinoma tissues using laser capture microdissection (LCM) together with quantitative reverse transcriptase-PCR (RT-PCR) and immunohistochemistry (8, 9). The effects of testosterone on both A549 and LK87 cell proliferation, and the inhibitory effects of the aromatase inhibitor letrozole on cell proliferation, were subsequently evaluated. We also showed the production of estrogen as a result of conversion from testosterone in both A549 and LK87 cells.

In human breast carcinoma tissue, aromatase expression in both stromal and parenchymal cells was reported to be regulated by various factors such as cell-to-cell interactions and cytokines (9–12). Therefore, in this study, we examined the possible effects of isolated stromal cells from NSCLC tissues on expression of aromatase mRNA transcripts in NSCLC cell lines using a coculture system (9, 12). We then screened the expression of factors that have been reported to induce aromatase, such as interleukin-6 (IL-6), oncostatin M, and tumor necrosis factor-α (TNF-α), derived from stromal cells using cytokine antibodies arrays, and evaluated the effects of these cytokines on aromatase expression in A549 and LK87, respectively.

Materials and Methods

Laser capture microdissection

Six lung adenocarcinoma cases [female, A1–A3 (48, 65, and 77 years old); male, A4–A6 (53, 58, and 70 years old)] and three lung squamous cell carcinoma cases [male, S1–S3 (55, 76, and 60 years old)] were obtained from the patients in the Department of Thoracic Surgery at Tohoku University Hospital. Research protocols for this study were approved by the Ethics Committee of Tohoku University Graduate School of Medicine (no. 2008-444). Lung carcinoma tissues were rapidly embedded in Tissue-Tek optimal temperature compound (Sakura Finetechnical Co., Ltd.) and frozen sectioned at a thickness of 8 μm (9). We used 8 μm in this study because this thickness is generally considered to contain a monolayer of relatively homogeneous cell populations without contamination of other components (13). Approximately 5,000 cells were laser transferred from both carcinoma and intratumoral stromal cells under light microscopic evaluation (9). LCM was performed using mmi CellCut (MMI Molecular Machines & Industries AG). Total RNA was extracted using RNeasy Micro Kit (Qiagen GmbH), and a QuantiTect Reverse Transcription Kit (Qiagen) was used in the synthesis of cDNA.

Quantitative RT-PCR

Quantitative RT-PCR was carried out using the LightCycler System (Roche Diagnostics GmbH). The primer positions used in this study were as follows: RPL13A (NM_012423), forward 487–reverse 612 (9); aromatase (X13589), forward 691–reverse 806 (9). Aromatase mRNA levels in each case were represented as a ratio of RPL13A and evaluated as a percentage ratio (9).

Immunohistochemistry

Sequential frozen tissues, also used in the LCM analyses above, were used to examine the correlation between mRNA and protein in individual cellular compartments of the lung cancer tissues with immunohistochemistry using monoclonal antibody no. 677 (9, 14, 15). Evaluation of aromatase immunohistochemistry was performed based on staining intensity proportion scoring systems used for breast carcinoma tissues (15) with some modifications (9, 14). The approximate percentage of immunopositive cells (proportion score) were classified into the following four groups: 0, <1%; 1, 1–25%; 2, 25–50%; and 3, >50% immunopositive cells. The relative intensity of aromatase immunopositive cells was classified as follows: 0, no immunoreactivity; 1, weak; 2, moderate; and 3, intense immunoreactivity. Aromatase immunoreactivity was evaluated as a total score composed of the proportion score + relative immunoreactivity score. Results of immunohistochemistry were independently evaluated by two of the authors (Y.M. and K.A.).

Lung carcinoma cell lines and culture conditions

We used A549, LCSC#1, RERF-LC-OK, LK87, LK2, WI-26, BEAS-2B, NCI-H727, RERF-LC-AI, and LCAM1. The original tissue, source, and medium were summarized in Supplementary Table S1. Cells were maintained in each medium supplemented with 10% fetal bovine serum (FBS; JRH Biosciences Co.). MCF-7 was used as a positive control of aromatase. In our previous studies (7, 16), we showed that A549, provided by Cell Resource Center for Biomedical Research (Tohoku University, Sendai, Japan), did not have detectable levels of ERα and ERβ proteins and was unresponsive to estradiol treatment in reporter gene assay. A549 used in this study was the same stock as in the previous study (16). Estradiol and testosterone were obtained from Sigma-Aldrich. The ER blocker, ICI 182,780, and the androgen receptor (AR) blocker, hydroxyflutamide (flutamide), were obtained from Tocris Cookson Ltd. and Toronto Research Chemicals, Inc., respectively. The aromatase inhibitor, letrozole, was provided by Novartis Pharmaceutical Corporation. These materials were dissolved in 99.5% ethanol (Wako Pure Chemical Industries).

Cell proliferation assay

Both A549 and LK87 were used in the cell proliferation assay. The amounts of ERα, ERβ, and AR mRNA expression determined by quantitative RT-PCR were summarized in Supplementary Fig. S1A. The cells were cultured in phenol red–free RPMI 1640 (modified RPMI 1640, Sigma-Aldrich Co.) with dextran-coated charcoal (DCC)-FBS for 2 days before each experiment. The effects of steroids and drugs on these cell lines were evaluated using both anchorage-dependent and anchorage-independent cell proliferation assays (17).
Anchorage-dependent cell proliferation assay. The cells were seeded onto normal-adhesion 96-well plates (AGC TECHNO GLASS CO., LTD.) in phenol red–free medium containing 10% DCC-FBS. The cells were treated with steroids and test compounds for 24, 48, and 72 hours, respectively, when these cells were harvested and evaluated for cell proliferation using the WST-8 colorimetric assay (Cell Counting Kit-8; Dojindo Laboratories; ref. 18).

Anchorage-independent cell proliferation assay. The cells were cultured in low-adhesion 24-well plates (AGC TECHNO GLASS) in phenol red–free medium containing 10% DCC-FBS. The cells were then treated with steroids and test compounds for 3, 6, and 9 days, respectively, when these cells were harvested. The cell number was determined by using a cell counter (Sysmex CDA-500, Sysmex Corporation).

Steroid production assays
A549 and LK87 cells cultured onto 100-mm dishes were incubated at 37°C in phenol red– and FBS-free medium containing 100 nmol/L testosterone as substrates for 24 hours. The effects of 5 μmol/L flutamide and 10 nmol/L letrozole on steroid hormone production were also examined. The concentrations of estrone, estradiol, testosterone, and 5α-dehydrotestosterone (5α-DHT) were evaluated by liquid chromatography-electrospray tandem mass spectrometry analysis (ASUKA Pharma Medicals Co.; refs. 9, 19). All cells treated with steroids were counted by the cell counter. After addition of 100 μg of androstenedione-2H2 (C/D/N Isotopes) and estrone-13C4 and estradiol-13C4 (Hayashi Pure Chemical Industries), as internal standards, steroids were extracted with diethyl ether from the medium. In this study, we used liquid chromatography (Agilent 1100; Agilent Technologies, Inc.) coupled with an API 4000 triple-stage quadrupole mass spectrometer (Applied Biosystems, Inc.) operated with electron spray ionization in the positive-ion mode, and the chromatographic separation was performed on Cadenza CD-C18 columns (Imtakt Corporation). Steroid hormone levels in each case are presented as pg/mL/10^4 cells (estrogen) or ng/mL/10^4 cells (androgen).

Effects of stromal cells on aromatase expression in carcinoma cells
Intratumoral stromal cells. The primary stromal cells used in our present study were designated LK001S and LK002S, respectively, and isolated using collagenase treatment (20) from human lung carcinoma tissues. After tissue disaggregation with collagenase S-1 (Nitta Gelatin, Inc.) for 1 to 1.5 hours, these stromal cells grown to confluence were subsequently cultured in RPMI 1640 with 10% FBS. The outgrowth of these cells was detected at 3 days (LK002S) or 5 days (LK001S) of cell culture. These lung intratumoral stromal cells showed typical morphological features of fibroblasts under light microscopy. There were no morphologic changes and cell viabilities between the cells in passages 5 and 8 used in the experiment.

Coculture system. For physical separation of stromal and carcinoma cell lines, transwell cultures were established in 100-mm dishes using Transwell Permeable Supports (0.4-μm pore; Corning Incorporated; refs. 9, 12). Both A549 and LK87 cells were cultured in transwell chambers in the absence or presence of LK001S and LK002S cells and were placed at the bottom of the dishes. After 72 hours of cultivation using this coculture system, carcinoma cells were separated and the levels of aromatase mRNA expression were examined by real-time RT-PCR analysis as described above.

Conditioned medium. Both LK001S and LK002S were cultured in 155-cm² culture flasks. The culture medium was replaced by FBS and phenol red-free medium. After 24 hours, the conditioned medium (total 80 mL) was collected and concentrated to a total volume of 16 mL using Macrosep Centrifugal Devices (Pall Corporation). A549 cells were cultured onto a six-well plate in the medium containing 5%, 10%, and 20% condensed conditioned medium (CC-medium). After 72 hours of culture, aromatase mRNA levels were evaluated by real-time RT-PCR as described above.

Cytokine analysis
In this study, we used Human Cytokine Antibody Array 5 (RayBiotech, Inc.; refs. 21–23) to identify the cytokines released from stromal cells. Cytokine antibody membranes were incubated for 2 hours with 2 mL of CC-medium. The reacted membranes were then incubated for 1 hour with biotin-conjugated anti-cytokines and then developed with horse radish peroxidase–streptavidin and chemiluminescence. Protein dots were visualized with Las-1000 cooled CCD-camera chemiluminescent image analyzer (Fuji Photo Film Co., Ltd.).

Human recombinant TNF-α, IL-6, and oncostatin M were purchased from Wako Pure Chemical Industries. TNF-α (20 ng/mL), IL-6 (50 ng/mL), or oncostatin M (50 ng/mL) was added with 100 nmol/L dexamethasone (Wako Pure Chemical Industries). Aromatase mRNA expression was examined using quantitative RT-PCR described above. Aromatase protein expression was also evaluated using immunocytochemistry. Cells were grown directly on cover glasses (collagen type I–coated cover glass, round, 12 mm; AGC TECHNO GLASS) under the culture conditions described above. The relative abundance of reacted signals for aromatase was subsequently quantified as the optical density (OD) value by using the Multi Gauge software (Science Lab 2005 version 3.0; Fuji Photo Film).

Neutralization assay of cytokines
The monoclonal antibodies against TNF-α (clone 28401), IL-6 (clone 6708), and oncostatin M (clone 17001) used in neutralization assays were obtained from R&D Systems, Inc. Neutralizations were performed by adding 50 μg/mL of antibodies to 2 mL CC-medium described above for 24 hours on ice, with gentle shaking. All antibodies used were immobilized to magnetic Dynabeads Protein G (Invitrogen) by using 50 μg IgG for every 100 μL Dynabeads Protein G. After 2 hours of incubation on ice, the antibody-bound Dynabeads were collected using a magnet. The supernatants without antibody-bound Dynabeads, termed neutralizing CC-medium (NCC-medium), were also used for aromatase
expression assay. A549 cells were cultured in DCC-medium containing 20% NCC-medium. Aromatase mRNA expression levels were examined by quantitative RT-PCR after 72 hours of cell culture.

Statistical analysis
Results were expressed as mean ± SD. Statistical analysis was performed using the StatView 5.0 J software (SAS Institute, Inc.). The statistical difference of two groups was determined by Student's \( t \) test. Multiple comparisons were performed with Bonferroni/Dunn test or Dunnett’s test. A \( P \) value of <0.05 was considered to indicate statistical significance.

Results
Localization of aromatase mRNA and protein in lung carcinomas
In six lung adenocarcinoma cases, aromatase mRNA transcripts were detected in carcinoma cells from five cases and in stromal cells from two cases (Fig. 1A). Aromatase mRNA expression was predominantly detected in carcinoma or parenchymal cells. In case A3, a relatively low level of aromatase mRNA was detected only in stromal cells. In the same cases evaluated by aromatase LCM/PCR analysis, aromatase immunoreactivity was detected in the cytoplasm of carcinoma cells but not stromal cells from all six cases examined in this study (Fig. 1B). Aromatase immunoreactivity was not detected in all the stromal cells of these six cases. The cumulative or total aromatase immunohistochemistry score of each case was as follows: case A1, 3; A2, 6; A3, 4; A4, 6; A5, 2; A6, 0. The results between LCM/PCR analysis and immunohistochemistry of aromatase were consistent in five cases, but a discrepancy was detected in case A3. In three lung squamous cell carcinoma cases (S1, S2, and S3), the levels of aromatase mRNA transcripts were below the detection limits in both intratumoral carcinoma and stromal cells (data not present). In addition, no aromatase immunoreactivity was detected in these three cases of squamous cell carcinoma (data not present).

Figure 1. Aromatase expression in lung adenocarcinoma tissues and lung carcinoma cell lines. A, aromatase mRNA level in each case (cases A1–A6) of lung adenocarcinoma (white columns) and stromal (black columns) cells. B, aromatase immunohistochemistry for each case (cases A1–A6) of lung adenocarcinoma tissues. Aromatase immunoreactivity was detected in carcinoma cells. IHC, immunohistochemistry. C, aromatase levels in lung carcinoma and normal lung cell lines. MCF-7, breast carcinoma cell line.
**Aromatase mRNA expression levels in human lung carcinoma cell lines**

**Aromatase** mRNA was detected in all lung carcinoma cell lines except for LCSC#1 examined in this study (Fig. 1C). Relatively high levels of aromatase mRNA, with the same level as in MCF-7, were detected in both LK87 and NCI-H727. We therefore used the relatively higher aromatase mRNA—expressing LK87 and low aromatase mRNA—expressing A549 in further evaluations. The level of aromatase mRNA expression in lung carcinoma cell lines was ~10 times lower than that in intratumoral carcinoma cells of lung carcinoma tissues.

**Effects of estrogen and aromatase on cell proliferation in LK87**

In the anchorage-independent assay, the number of LK87 cells was significantly increased following estradiol treatment (1 nmol/L–1 μmol/L) for 6 days (Fig. 2A). Estradiol-induced cell proliferation was significantly inhibited ($P < 0.001$) by ICI 182,780 (1 μmol/L) treatment, with cell proliferation nearly comparable with that at the basal level (Fig. 2A). Estradiol- or testosterone-mediated cell proliferation was not detected in A549 cells treated with 1 nmol/L to 1 μmol/L estradiol or testosterone (Supplementary Fig. S2A and B). Testosterone treatment (1 nmol/L–1 μmol/L) significantly decreased the number of LK87, which was also inhibited by treatment with the specific androgen receptor blocker flutamide (5 μmol/L; Fig. 2B). When LK87 cells were treated with testosterone (1 nmol/L–1 μmol/L) and 5 μmol/L flutamide simultaneously for 3, 6, and 9 days, the number of the cells was significantly increased by these treatment compared with that of the control level (Fig. 2B). This increased cell proliferation was significantly inhibited by addition of ICI 182,780 (1 μmol/L; Fig. 2C) or the aromatase inhibitor letrozole (10 nmol/L; Fig. 2D). ICI 182,780, flutamide, and letrozole alone did not significantly change the cell proliferation of LK87 cells (Supplementary Fig. S2C).
when anchorage-dependent/WST-8 assay was used in these experiments (Supplementary Fig. S1B–E).

**Estrogen and androgen production in LK87**

Following testosterone treatment (288.4 mg/mL), the conversion rate into estradiol and estrone in LK87 was 5.95 ± 0.55 and 0.20 ± 0.02 mg/mL/10^6 cells (mean ± SD, n = 3), respectively (Fig. 3A). Estrogen production was significantly decreased by 5 μmol/L flutamide alone or 10 nmol/L letrozole + 5 μmol/L flutamide treatment (Fig. 3A). The concentration of testosterone and 5α-DHT in LK87 treated with 10 nmol/L testosterone was 10.43 ± 0.79 and 1.41 ± 0.21 mg/mL/10^6 cells (mean ± SD, n = 3), respectively (Fig. 3B). These androgen productions were significantly increased by 10 nmol/L letrozole + 5 μmol/L flutamide treatment (Fig. 3B).

**Effects of coculture with stromal cells on aromatase mRNA expression in LK87 and A549**

The results of the effects of the coculture of stromal cells on LK87 and A549 aromatase mRNA expression levels are summarized in Fig. 4A. Aromatase mRNA levels of LK87 following coculture with LK002S were significantly higher than those in monocultured LK87. Aromatase mRNA levels in A549 (after coculture with both LK001S and LK002S) were also significantly higher than those in monocultured A549 cells.

**Effects of cytokines derived from stromal cells on aromatase mRNA expression in LK87 and A549**

The results of the expression of IL-6, oncostatin M, and TNF-α derived from lung intratumoral stromal LK001S and LK002S cells are summarized in Fig. 4B. In LK002S, IL-6, oncostatin M, and TNF-α were all detected by cytokine array analysis. The levels of all these three cytokines were increased by coculture with both A549 and LK87 cells. Both IL-6 and oncostatin M were also detected in the cells derived from LK001S. However, TNF-α was under the level of the detection limit in LK001S. The level of IL-6 was increased following coculture with A549 and decreased by coculture with LK87. The level of oncostatin M was increased by coculture with both A549 and LK87 cells.

Addition of CC-medium (10% and 20%) from both LK001S and LK002S resulted in significant increment of aromatase mRNA levels in A549 (Fig. 4C). The aromatase mRNA level was significantly decreased by addition of NCC-medium of IL-6 (LK001S) or both IL-6 and oncostatin M (LK002S) compared with that of CC-medium alone (Fig. 4D). There were no differences on the degrees of aromatase mRNA induction.
between NCC-medium of TNF-α and CC-medium alone in A549 cells (Fig. 4D).

The effects of these cytokine treatments on LK87 and A549 aromatase levels are summarized in Fig. 5. In LK87 cells, treatment with oncostatin M + dexamethasone significantly increased aromatase mRNA transcript levels, but the treatment of dexamethasone alone did not influence aromatase expression in LK87 (Fig. 5A). In A549 cells, the aromatase mRNA transcript level was significantly increased by the treatment of dexamethasone alone compared with that at the control level (Fig. 5A). Treatment of both oncostatin M + dexamethasone and IL-6 + dexamethasone significantly
increased aromatase mRNA transcript levels compared with the treatment of dexamethasone alone (Fig. 5A). Treatment of TNF-α did not significantly influence aromatase mRNA transcript levels in both LK87 and A549. An example of immunoreacted glasses of aromatase in A549 at the macrolevel is illustrated in Fig. 5B. The immunoreactivity of aromatase was relatively high in 50 ng/mL oncostatin M treatment with or without dexamethasone. Low immunoreactivity of aromatase was detected in A549 treated with 5 ng/mL oncostatin M or 50 ng/mL IL-6 treatments. An

Figure 5. Effects of IL-6, oncostatin M, and TNF-α on aromatase expression in lung carcinoma cell lines. A, effects of IL-6, oncostatin M, and TNF-α treatments with dexamethasone (DEX) on aromatase mRNA level in LK87 and A549 cells. *, P < 0.05 versus control (CTL); †, P < 0.05 versus dexamethasone alone (CTL/DEX). B, effects of IL-6 and oncostatin M treatments with or without dexamethasone on immunoreactivity of aromatase in A549 cells. An example of immunoreacted glasses of aromatase is shown at the macro level. DEX (−), without dexamethasone; DEX (+), with dexamethasone (ng/mL). C, immunocytochemistry of aromatase in A549 cells treated with both oncostatin M and dexamethasone. An example of immunoreacted glasses of aromatase at the microscopic level is illustrated. The immunoreactivity of aromatase was detected in 5 ng/mL (c) and 50 ng/mL (d) oncostatin M with dexamethasone treatment. a, control (dexamethasone alone); b, 0.5 ng/mL oncostatin M with dexamethasone. D, relative immunointensity of aromatase in A549 cells treated with both oncostatin M and dexamethasone. The relative abundance of reacted signals for aromatase was subsequently quantified as the OD value. *, P < 0.05 versus 0 (0 ng/mL oncostatin M/dexamethasone or 0 ng/mL oncostatin M alone).
Aromatase has been reported to be predominantly expressed in intratumoral stromal cells and adipocytes of human breast carcinoma tissues (9, 10, 24). In lung carcinoma, however, several studies showed aromatase protein in parenchymal cells but not in stromal cells (4, 6, 25). There have been controversies as to the localization of intratumoral aromatase in human breast carcinoma (3) because many results have been obtained by immunohistochemistry, which were influenced by the nature of antibodies and specimen preparation. In our present study, the results of combined LCM and RT-PCR analysis of aromatase showed that in lung carcinoma, aromatase mRNA/protein was detected predominantly in parenchymal cells of lung cancer tissues in contrast to its localization in human breast carcinoma evaluated by the same methodology (8). Similar localization of aromatase has also been reported in colon (26), gastric (27), and oral squamous cell carcinomas (28). Aromatase immunoreactivity was also reported to be predominantly present in intratumoral stromal cells in endometrial (29, 30) and prostatic (31) carcinomas as well as in breast carcinoma (9, 10, 13). These data all suggest that stromal cells in classic estrogen-dependent malignancies, such as breast and endometrial cancers, are all associated with abundant overexpression of aromatase.

The amount of aromatase mRNA in lung carcinoma cell lines was \( \sim 10 \) times lower than that detected in intratumoral stromal cells of breast carcinomas (9). In our present study, a relatively high rate of conversion into estradiol was detected in LK87 following the treatment of testosterone. LK87 used in this study has relatively high levels of ER\( \beta \) and estrogen-dependent cell proliferation. This finding above all suggests that "functional" aromatase and subsequent estrogen-dependent cell proliferation pathways exist in LK87. In our present study, we also showed that the AR blocker flutamide also exerted inhibitory effects on estradiol production as a result of conversion from testosterone. Androgens were reported to stimulate aromatase expression/activity through an AR-dependent pathway in rodent brains (32, 33). Therefore, aromatase expression was inhibited by blocking of AR-dependent transcription in lung carcinoma cell lines. Intratumoral concentration of 5α-DHT in breast carcinoma tissues following treatment of aromatase inhibitor was significantly higher than in those without treatment (34). In our present study, 5α-DHT production in LK87 was also significantly increased by letrozole with flutamide treatment. Aromatase inhibitors are therefore considered to increase in situ availability of androgens in lung carcinoma tissues as well as in breast carcinoma, possibly by increasing testosterone concentrations. In breast carcinoma, androgens have been shown to predominantly exert anti-proliferative effects through an AR-dependent pathway (35). Therefore, all of these findings suggest that lung cancer patients, who expressed aromatase, ER (ER\( \alpha \) and/or ER\( \beta \)), and possibly AR, may benefit more from aromatase inhibitor therapy. However, in contrast to estrogen, the effects of androgens on lung carcinoma cells have remained largely unclear.

There was a "discrepancy" in terms of amounts of aromatase expression between the tissues of the patients and lung carcinoma cell lines. This discrepancy is considered to be due to the conditions of cell culture such as culture period and cell-to-cell contact. In addition, results from our coculture analyses in lung and breast carcinoma (9) cells suggest that this discrepancy is partly due to the lack of interaction of carcinoma cells and stromal cells. Therefore, in this study, we focused on the effects of cytokines derived from stromal cells on aromatase induction in lung carcinoma cells. However, it is true that several investigators showed that soluble factors, including platelet-derived growth factor and transforming growth factor \( \beta \), derived from lung carcinoma cells induced differentiation and cell proliferation of fibroblastic stromal cells (36). Therefore, aromatase-inducible cytokines secreted from stromal cells of carcinoma tissues may be under the control of an interaction with carcinoma cells in the lung cancer microenvironment.

The results of previously reported studies all showed that various aromatase-stimulating factors (10, 11, 37, 38) were released from parenchymal or carcinoma cells, which subsequently resulted in the upregulation of aromatase expression in stromal cells of human breast carcinoma tissues. In our present study, aromatase mRNA/protein in A549 was also shown to be induced by both IL-6 and oncostatin M treatments. In adipose stromal cells, the effects of IL-6, IL-11, and oncostatin M were all reported to be mediated through the Jak/signal transducer and activator of transcription 3 signaling pathway (39), and interferon-\( \gamma \) activation site element was located in the upstream of aromatase promoter L4 (39). We also showed in our present study that relatively high levels of cytokines such as IL-6, TNF-\( \alpha \), and oncostatin M were detected in conditioned medium obtained from LK001S and LK002S cultures. In addition, the results of these cytokine analyses also showed that the levels of IL-1 and IL-11 secretion from stromal cells were below the detection limits (data not present). These results also suggest that both IL-6 and oncostatin M secreted from stromal cells were required to induce aromatase expression in the lung intratumoral microenvironment. However, neutralization of both IL-6 and oncostatin M did not necessarily decrease aromatase expression to the control levels. Further investigations are required to clarify the mechanisms of regulation of intratumoral aromatase in the human lung carcinoma microenvironment.

In summary, some human lung carcinomas are considered to be estrogen-dependent carcinomas, and aromatase plays a pivotal role in intratumoral estrogen synthesis. In
lung carcinoma, this aromatization mainly occurs in carcinoma cells under the influence of various cytokines derived from intratumoral fibroblastic stromal cells. The results of our previous study (9) showed that aromatase inhibitors such as letrozole and exemestane were more effective on the aromatase enzymatic activity of breast carcinoma MCF-7 cells than in stromal 32N cells alone. Therefore, aromatase inhibitors used in ER-positive breast carcinoma patients may also be effective in NSCLC patients associated with intratumoral aromatase.

Disclosure of Potential Conflicts of Interest

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