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Lysyl Oxidase Oxidizes Basic Fibroblast Growth Factor and Inactivates Its Mitogenic Potential

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Abstract Lysyl oxidase (LO) plays a central role in the crosslinking of collagen and elastin in the extracellular matrix. Here we demonstrate that basic fibroblast growth factor (bFGF), a polypeptide which regulates proliferation, differentiation, and migration of a variety of cell types, is a substrate of LO. The oxidation of lysine residues in bFGF by LO resulted in the covalent crosslinking of bFGF monomers to form dimers and higher order oligomers and dramatically altered its biological properties. Both the mitogenic potential and the nuclear localization of bFGF were markedly inhibited in the Swiss 3T3 cells upon its oxidation by LO. NIH 3T3 IgBNM 6-1 cells (6-1 cells) overexpress bFGF which participates in an autocrine mechanism accounting for the transformation of these cells into a tumorigenic state. Exposure of the 6-1 cells to nanomolar concentrations of LO in culture oxidized lysine and generated crosslinkages in bFGF within the cell and markedly reduced proliferative rates. The lack of LO expression has been correlated with hyperproliferative cell growth, while this enzyme has been identified as a suppressor of *ras*-induced tumorigenesis. The present results illustrate a mechanism by which LO can depress normal and transformed cell growth. J. Cell. Biochem. 88: 152–164, 2003.

Key words: lysyl oxidase; bFGF; inhibition of proliferation; oxidation; growth factors

Lysyl oxidase (EC 1.4.3.13), a copper-dependent amine oxidase expressed and secreted by fibrogenic cells, initiates the covalent crosslinking of collagen and elastin in the extracellular space by oxidizing specific lysine residues in these proteins to form peptidyl α-aminoadipic-δ-semialdehyde. These peptidyl aldehyde residues can spontaneously condense with vicinal peptidyl aldehydes or unreacted lysines to generate the covalent crosslinkages which stabilize and insolubilize polymeric collagen or elastin fibers. Thus, lysyl oxidase (LO) plays a central role in the morphogenesis and repair of connective tissues of the cardiovascular, respiratory, skeletal, and other systems of the body. Increased LO activity is associated with fibrotic diseases such as atherosclerosis while decreased LO activity accompanies disorders of copper metabolism as occur in Menke's syndrome [Kagan, 1986; Gacheru et al., 1993]. In addition to its collagen and elastin substrates, LO can also oxidize lysine residues in various globular proteins which have basic isoelectric points such as histone H1 [Kagan et al., 1984]. Indeed, recent evidence indicates that LO may play critical roles in biology in addition to its role in stabilizing the extracellular matrix (ECM). This catalyst has been demonstrated to suppress *ras*-induced tumorigenesis [Kenyon et al., 1991], it has been shown to be chemotactic for monocytes and vascular smooth muscle cells [Lazarus et al., 1995; Li et al., 2000], it is present within cell nuclei [Li et al., 1997] and appears to modulate the packing state of nuclear chromatin [Mello et al., 1995].

Basic fibroblast growth factor (bFGF) is present in all organs, solid tissues, tumors, and cultured cells examined [Rifkin and Moscatelli, 1989] and is one of a family of at least 19

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heparin-binding polypeptides which regulate cell proliferation, migration, and differentiation [Rifkin and Moscatelli, 1989; Nishimura et al., 1999; Nugent and Iozzo, 2000]. This polypeptide is also implicated in a number of pathological states including wound healing, inflammation, angiogenesis, and tumor growth [Rifkin and Moscatelli, 1989; Nugent and Iozzo, 2000]. Human bFGF is produced as four isoforms, i.e., 18, 22, 22.5, and 24 kDa molecules that are translated from alternative initiation sites within a single mRNA species [Florkiewicz and Sommer, 1989; Prats et al., 1989]. These isoforms differ in their amino terminal extremities, which confer different intracellular localizations and functions [Stachowiak et al., 1996; Arese et al., 1999]. The 18-kDa form is distributed in the cytoplasm and can modulate cell migration and cell proliferation, while the higher molecular weight 22, 22.5, and 24 kDa forms are localized in the nucleus and appear to modulate processes during cell division [Bikfalvi et al., 1995; Stachowiak et al., 1996; Arese et al., 1999]. Although bFGF lacks a consensus signal sequence as in nascent proteins destined for secretion, the 18-kDa isoform can be secreted, although by a pathway independent of the endoplasmic reticulum [Mignatti et al., 1992]. bFGF has been visualized in ECM of different tissues [Rifkin and Moscatelli, 1989]. Extracellular bFGF forms a complex with and is stabilized by heparan sulfate proteoglycan (HSPG) in the ECM [Rifkin and Moscatelli, 1989; Nugent and Iozzo, 2000]. Moreover, membrane bound HSPG act as binding sites for bFGF, which participate with bFGF tyrosine kinase receptors to initiate intracellular signaling and biological response [Nugent and Edelman, 1992; Schlessinger et al., 1995].

Human bFGF is a basic protein ($pI > 9.0$) containing 14 lysine residues within a total of 155 amino acids [Burgess and Maciag, 1989; Rifkin and Moscatelli, 1989]. Certain of these lysines are accessible at the protein surface, among which K26, K125, and K135 [residues are numbered as in Eriksson et al., 1991] act as heparin binding sites [Eriksson et al., 1991; Faham et al., 1996]. In view of the preference of LO for lysine residues within basic protein substrates [Kagan et al., 1984] and since LO could encounter bFGF in the extracellular space, we have investigated the possibility that bFGF can be oxidized by LO and assessed the biological consequences of such an event. We observed that LO readily oxidized bFGF and markedly reduced bFGF-dependent growth of normal and transformed cells.

MATERIALS AND METHODS

Materials

Sodium homovanillate, β-aminopropionitrile (BAPN), propidium iodide, horseradish peroxidase (HRP), RNAse, fluoroscein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG, and rhodamine-coupled goat anti-rabbit IgG were purchased from Sigma Chemical Co. (St. Louis, MO). Human recombinant bFGF (18 kDa) was obtained from Scios, Inc. (Mountain View, CA). ^{125}I-Bolton Hunter reagent was from Dupont NEN (Boston, MA). Polyclonal antibody against ERK1/2 and monoclonal antibody against bFGF were from Santa Cruz Biotech (Santa Cruz, CA). Monoclonal antibody against phosphorylated-ERK 1/2 was from New England BioLabs (Beverly, MA). All tissue culture products were from GIBCO (Grand Island, NY).

Enzyme Purification and Assay

Calf aorta LO (32 kDa) was isolated as described [Bedell-Hogan et al., 1993]. The specific activity of the enzyme preparation used in this study was 7.5×10^5 cpm of [^{3}H]H_2O release/mg protein in 2 h at 37°C, using 1.25×10^5 cpm of [^{3}H]tropoelastin substrate per assay [Bedell-Hogan et al., 1993].

Cell Culture

Swiss 3T3 fibroblasts, plated at $2–3 \times 10^5$ cells per 60-mm dish, were cultured in 10% fetal bovine serum/Dulbecco's Modified Eagle's Medium (FBS/DMEM) for 20 h, growth-arrested by incubation in 0.3% FBS/DMEM for 3 days, and then used for experiments as described [Li et al., 1995]. The NIH 3T3 IgBNM6-1 cell line [Rogelj et al., 1988, 1989] used in this study overexpresses bFGF and was derived from NIH 3T3 fibroblasts which had been transfected with bFGF cDNA [Rogelj et al., 1988] encoding 18 kDa bFGF fused at its second amino acid residue to an amino terminal immunoglobulin signal peptide of 19 amino acids. The 18-kDa bFGF produced in excess by these cells was not secreted, but remained associated with the cells [Rogelj et al., 1988]. Aliquots containing 2×10^4 6-1 cells were seeded in 35-mm dishes containing 2 ml 10% FBS/DMEM. The medium was then changed to 0.3% FBS/

DMEM in the presence or absence of LO, of BAPN, an irreversible inhibitor of LO activity [Kagan, 1986], or combinations at the indicated concentrations. Cells were harvested by incubation with trypsin and counted with the aid of a hemocytometer after 6 days of incubation.

Fluorometric Assay for LO-Dependent H_2O_2 Release

The reaction of LO with bFGF as a substrate was assessed by an HRP-coupled fluorescence assay for H_2O_2 production [Trackman et al., 1981]. Reaction mixtures contained 0.25 mg of sodium homovanillate, 40 μg of HRP, and bFGF at various concentrations in 0.05 M sodium borate buffer, pH 8.2, in a final volume of 2 ml. Assays were initiated by the addition of LO to reaction mixtures. H_2O_2 release was continuously monitored at excitation and emission wavelengths of 315 and 425 nm, respectively, at a constant temperature of 55 or 37°C, as specified, in the thermostatted cuvette chamber of an SLM Aminco Bowman Series 2 Luminescence Spectrometer. All activity data was corrected for background rates of H_2O_2 release determined in complete assay mixtures supplemented with 200 μM BAPN. Standard curves relating H_2O_2 concentration to fluorescence units were established by adding aliquots of freshly titrated H_2O_2 to assay mixtures (2 ml) containing borate buffer, HRP and homovanillic acid.

Electrophoresis, Autoradiography, and Western Blotting Analyses

To determine whether bFGF oxidation by LO resulted in the formation of crosslinked polymers of the growth factor, human recombinant bFGF (18 kDa) was iodinated using the method described by the commercial supplier (New England Nuclear, Boston, MA). ^{125}I-Labeled bFGF (5 μg) was incubated with 4 μg of LO in 0.1 M sodium borate buffer, pH 8.2, in a final volume of 100 μl, at 37°C for 1 h. The reaction was quenched by the addition of 100 μM BAPN followed by incubation at 37°C for 30 min. Aliquots of the incubated reaction mixtures were analyzed by SDS–PAGE (15%) and autoradiography as described [Li et al., 1995]. For Western blot analysis of MAP kinase activation, control and treated cells were lysed in RIPA buffer [1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethanesulfonyl fluoride in phosphate-buffered saline (PBS)] according to the instructions of the RIPA lysis kit (Santa Cruz BioTech, Santa Cruz, CA). The collected lysates were passed through a 21-gauge needle ten times and centrifuged. Aliquots of supernatants containing equal amounts of protein (25 μg) were analyzed by SDS–PAGE, and the protein bands were then transferred to a nitrocellulose membrane. The blots were probed by incubation with a primary antibody specific for ERK1 and ERK2 or their phosphorylated forms and then with a secondary antibody conjugated with HRP. To examine the expression and intracellular forms of bFGF in 6-1 cells, control and treated cells were washed twice with PBS, incubated for 5 min at 37°C with 0.25% trypsin/1 mM EDTA in a balanced salt solution (GIBCO) to detach the cells and to release susceptible plasma membrane-bound macromolecules. Detached cells were suspended in 10% FBS/DMEM, centrifuged, washed again in PBS, and then lysed in the RIPA buffer. The cell lysates were then processed for Western blotting using the primary monoclonal bFGF antibody and the HRP-coupled second antibody. Reactive bands were detected by chemiluminescence.

Fluorescence-Activated Cell Sorting (FACS) Analysis

Growth-arrested Swiss 3T3 cells were incubated for 20 h at 37°C with or without bFGF, LO, BAPN, or their combinations at the concentrations indicated. The outcomes of the experiments were the same regardless of whether or not the LO, BAPN, and/or bFGF components were preincubated with each other for 15 min at 37°C prior to addition to the cell cultures. After incubation, cells were washed, suspended by treatment with trypsin, washed again and fixed in 0.5% formaldehyde/0.1% Triton X-100/PBS for 15 min on ice followed by incubation of samples in 10 μg propidium iodide and 20 U RNAse per ml of PBS for 60 min in the dark for DNA staining. The fluorescence intensities of each sample of 10,000 cells in G_1, S, and G_2/M phases were measured by flow cytometry at the FL2 setting of the FACS instrument (Becton-Dickinson Corp.), as described [Darzynkiewicz, 1994].

Fluorescence Microscopy

Growth-arrested Swiss 3T3 cells on coverslips were incubated for 1 h at 37°C with or

without bFGF, LO, BAPN, or combinations thereof, as specified. The cells were fixed in 3.7% formaldehyde/0.2% Triton X-100/PBS, blocked with 1% bovine serum albumin in PBS, and incubated with a monoclonal anti-bFGF antibody for 45 min at room temperature. Control cells were incubated in the absence of anti-bFGF to develop negative controls. Following thorough washing, the coverslips were overlaid with FITC-conjugated rabbit anti-mouse IgG and incubated for an additional 45 min at room temperature. In addition, growth-arrested 6-1 cells on coverslips were also stained with polyclonal anti-LO antibody followed by the rhodamine-coupled goat anti-rabbit IgG. The intracellular distribution and nuclear localization of bFGF or LO were examined under a Nikon fluorescence microscope as described [Li et al., 1997].

LO Uptake by 6-1 Cells

Cells grown on coverslips were exposed to FITC-conjugated LO (0.16 μg/ml) prepared according to the manufacturer's instructions (Molecular Probe, Eugene, OR). At various times of incubation, cells were fixed and assessed for internalized LO under the fluorescence microscope.

RESULTS

bFGF Oxidation by LO In Vitro

The oxidation of a primary amine substrate by LO yields stoichiometric amounts of the corresponding aldehyde, hydrogen peroxide, and ammonia, as shown: $RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + H_2O_2 + NH_3$ [Kagan, 1986]. To assess the substrate potential of bFGF, we monitored LO-dependent H_2O_2 release in assays using bFGF as the sole substrate, initially using the optimum temperature for LO activity of 55°C [Trackman et al., 1981]. As shown in Figure 1A, incubation of 0.16 μM LO with 0.23, 0.45, or 0.9 μM concentrations of 18 kDa human bFGF resulted in increased rates of H_2O_2 release with increasing concentrations of bFGF (solid curves a, b, and c). The curves exhibited an initial lag phase before the maximum rate developed following which the release of H_2O_2 ceased as evidenced by a plateau region seen in each curve. Introduction of additional aliquots of functional LO within the plateau phase of the curves did not cause additional H_2O_2 release, indicating that the plateaus represent maximum degrees of lysine oxidation in bFGF. The molar ratios of H_2O_2 released per mole of bFGF present at the plateau regions were 14.1, 14.6, and 14.2 for LO reacted with 0.23, 0.45, and

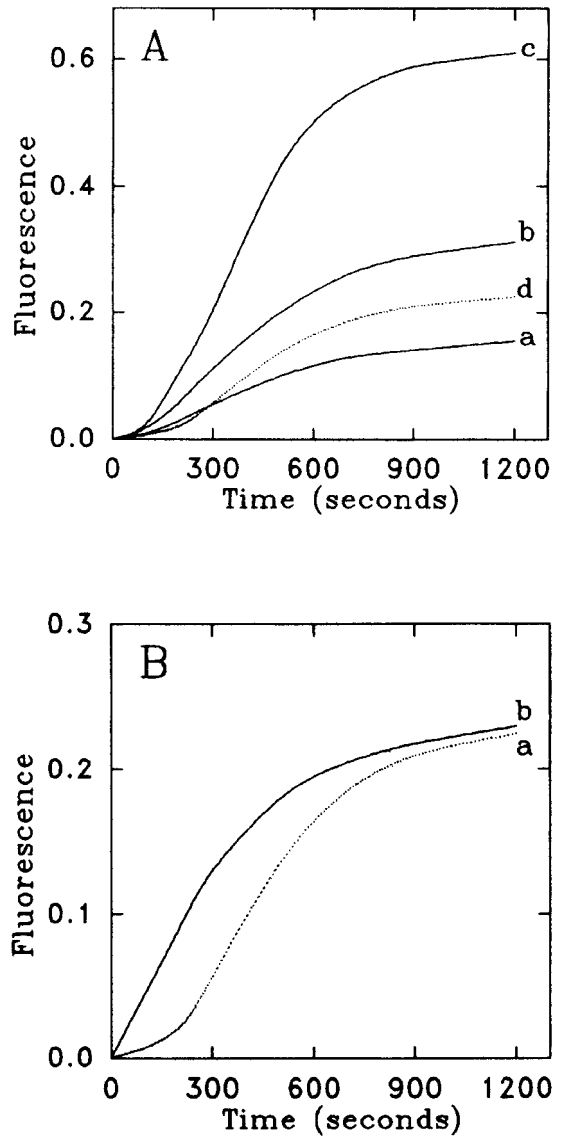


Fig. 1. Oxidation of bFGF by lysyl oxidase (LO) in vitro. **A**: Dose- and temperature-dependent responses. Solid curves represent the time course of H_2O_2 release at 55°C in the reaction of 0.16 μM LO with 0.23 (**a**), 0.45 (**b**), and 0.9 (**c**) μM bFGF, respectively. Dotted curve (**d**) represents the time course of H_2O_2 release in the reaction of 0.16 μM LO with 0.9 μM bFGF at 37°C. **B**: Effects of thermal preincubation of bFGF. The reaction mixture containing 0.9 μM bFGF received 0.16 μM LO within 30 s of the addition of bFGF (dotted curve **a**) or after preincubation of the bFGF in the reaction mixture for 5 min at 37°C (solid curve **b**). In each case, H_2O_2 release was monitored at 37°C. Note that plots of enzyme activity in (A) and (B) have been corrected for the background release of H_2O_2 obtained in the presence of 200 μM BAPN.

0.9 μM bFGF, respectively, yielding a mean value of 14.3 ± 0.3, consistent with the full complement of 14 lysine residues in each molecule of bFGF. Performing the assays at the physiological temperature of 37°C with 0.9 μM bFGF (dotted curve d) yielded a maximum of 5.3 moles of H_2O_2 released per mole of bFGF compared to the molar ratio of 14.3 ($[H_2O_2]$/[bFGF]) obtained at 55°C from the same concentration of the growth factor (compare curves c and d).

The initial lag observed in the plots relating oxidation to time was investigated. It was assumed that this lag was due to changes in the conformation and/or state of aggregation of bFGF induced by transferring the ice-cold stock solutions of bFGF to the 55° or 37°C assay temperatures. To test this possibility, 0.9 μM bFGF was pre-equilibrated in the reaction mixture lacking LO enzyme for 5 min at 37°C, following which the assay was initiated by the addition of 0.16 μM LO. The lag was eliminated under these conditions (Fig. 1B, curve b). In this case, hydrogen peroxide was immediately released at a rate corresponding to the maximum rate seen after the lag phase in an assay in which the bFGF substrate had not been thermally equilibrated prior to the addition of LO (Fig. 1B, curve a). The lag phase was also eliminated by pre-equilibration of bFGF at 55°C (not shown). Changes in the accessibility of lysine residues in bFGF to LO appear to reflect changes in the conformation and/or state of aggregation of bFGF upon warming and diluting the cold stock solution to the concentration and temperature of the assay.

Crosslinking of bFGF monomers as a result of its oxidation by LO was assessed by incubating 2.8 μM ^{125}I-bFGF with 1.25 μM LO in 0.1 M sodium borate, pH 8.2, for 1 h at 37°C. Samples were analyzed by SDS–PAGE and the resultant band positions visualized by autoradiography [Li et al., 1995]. As shown in Figure 2, incubation of bFGF with LO induced the appearance of covalent dimers, oligomers, and higher polymers (lane 2) derived from bFGF monomers (lane 1). The dimer of bFGF is a prominent product of the growth factor oxidized by LO (lane 2). The formation of covalently linked polymeric forms of bFGF was inhibited by BAPN (not shown). Clearly, bFGF is a productive substrate for LO and forms crosslinked-polymers as a result of the oxidation of peptidyl lysines by LO.

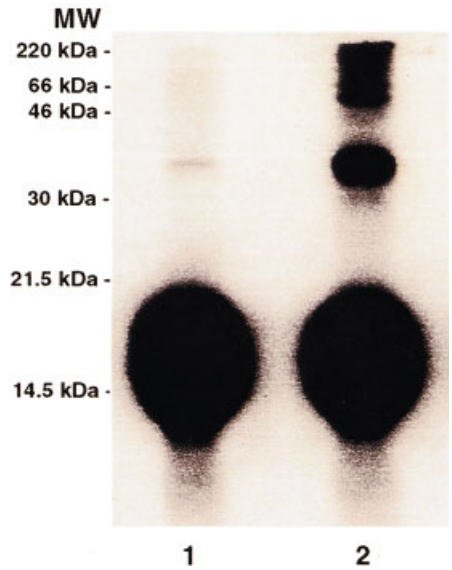
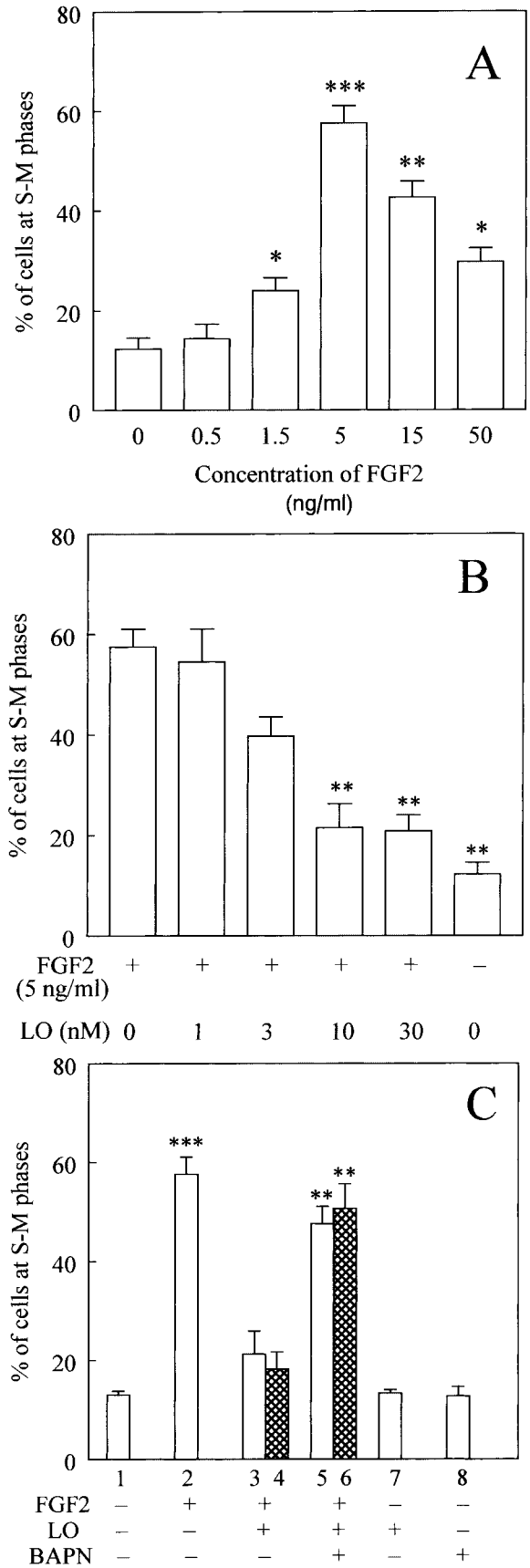


Fig. 2. LO-catalyzed crosslinking of ^{125}I-labeled bFGF revealed by SDS–PAGE and autoradiography. **Lane 1**: ^{125}I-bFGF (5 μg); **lane 2**, ^{125}I-bFGF (5 μg) + LO (4 μg). The relative electrophoretic mobilities of pre-stained molecular weight standards are indicated under MW.

Oxidation of bFGF by LO Inhibits Its Effects on Cell Cycle Progression and MAP Kinase Phosphorylation

As noted, specific lysine residues have been implicated in bFGF function [Eriksson et al., 1991; Faham et al., 1996]. Thus, we evaluated the activity of native and LO-oxidized bFGF on cell cycle progression using FACS analysis by previously defined methods [Darzynkiewicz, 1994]. As shown in Figure 3A, bFGF, alone, promoted the progression of growth-arrested Swiss 3T3 fibroblasts from the G_0 and G_1 to the S and G_2/M phases of the cell cycle. The maximum effect was seen at 5 ng/ml bFGF (0.28 nM) where the relative number of cells entering the S–M phases was 4.7-fold of that of the control. Cell cycle progression induced by bFGF was inhibited by incubation of the growth factor with increasing concentrations of LO (Fig. 3B). The degree of stimulation obtained with 0.28 nM bFGF was decreased by 80% in the presence of 10 nM (0.32 μg/ml) LO. As shown in Figure 3C, the addition of BAPN together with LO to the cell culture effectively prevented LO from inhibiting bFGF-induced cell cycle progression (compare bars 3 and 5). Inhibition of the stimulation of growth by bFGF was also seen if LO was preincubated with bFGF for 15 min prior to addition of this enzyme substrate mixture to the



cell culture [(compare bar 3 (not preincubated) with bar 4 (preincubated)]. Inclusion of BAPN in the preincubation of LO with bFGF prior to addition of this mixture to the cultures also prevented the LO-dependent inhibition of growth stimulation by bFGF [compare bar 5 (not preincubated) and bar 6 (preincubated) in Fig. 3C]. Clearly, the inhibition by LO of bFGF-stimulated cell cycle progression requires the catalytic function of LO. It should be noted that Trypan blue exclusion assays revealed that neither the control Swiss 3T3 cells nor the Swiss 3T3 cells incubated with 1, 3, 10, or 30 μM LO lost more than 5–6% of their initial levels of viability, during the 20 h period of incubation in DMEM/0.3% FBS. Thus, incubation with this enzyme at the indicated concentrations does not exert irreversible, toxic effects on these cultures.

Since activation of MAP kinases by bFGF is one of the earliest intracellular, mitogenic signaling events in response to bFGF [Maher, 1999], we investigated the effects of LO on bFGF-stimulated MAP kinase phosphorylation in Swiss 3T3 cells by Western blot analysis using an antibody specific for phosphorylated ERK1 and ERK2. The total amount of ERK1 and ERK2 MAP kinases within the same cell extracts was determined by using an antibody that recognizes both the phosphorylated and non-phosphorylated forms of ERK1 and ERK2. As shown in Figure 4A, tyrosine phosphorylation of both 42- and 44-kDa ERKs was markedly increased after stimulation of the cells with 0.28 nM bFGF for 10 min (lane 2) in comparison to the control (lane 1). The level of phosphorylated MAP kinases was reduced by concomitant treatment of cells with 0.28 nM bFGF and 10 nM

Fig. 3. LO modulation of bFGF-stimulated Swiss 3T3 cell cycle progression revealed by FACS analysis. **A**: Dose response of bFGF in cell cycle progression. **B**: LO inhibition of bFGF-stimulated cell cycle progression (the control represents cells treated with 5 ng/ml bFGF in the absence of added LO). **C**: Effects of inactivation of LO by BAPN on bFGF-stimulated cell cycle progression. LO, 10 nM; bFGF, 5 ng/ml; BAPN, 100 μM. Bars 3 and 5: LO and bFGF (±BAPN) were added to cell cultures simultaneously; bar 4: bFGF was preincubated with LO for 15 min at 37°C; bar 6: LO was inactivated by preincubation with BAPN for 15 min at 37°C. This mixture was then incubated with bFGF for an additional 15 min at 37°C and then was added to the cell cultures. Data represent the means (±SD) of three separate experiments each assessed with duplicate dishes. Asterisks indicate values significantly different from the control as determined by ANOVA analysis: $*P<0.05$, $**P<0.01$, $***P<0.001$.

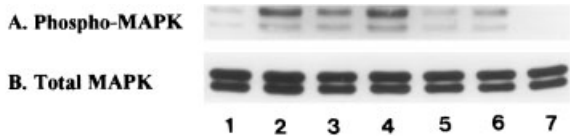


Fig. 4. Inhibition by LO of bFGF-stimulated MAP kinase phosphorylation. Growth-arrested cells were untreated (**lane 1**) or treated with 5 ng/ml bFGF (**lane 2**), 5 ng/ml bFGF + 10 nM LO (**lane 3**), 5 ng/ml bFGF + 10 nM LO + 100 μM BAPN (**lane 4**), 100 μM BAPN (**lane 5**), 10 nM LO (**lane 6**) or 200 μM genistein (**lane 7**) for 10 min. Cell extracts were prepared and equal amounts of protein (25 μg) were analyzed by SDS–PAGE. Immunoblotting was performed using antibodies specific for the active forms of ERK1 and ERK2 (**A**) and total ERKs (**B**).

LO (lane 3). The inhibitory effects of LO on bFGF activation of MAP kinase was abolished by inhibition of LO catalysis by the inclusion of 100 μM BAPN (lane 4). Neither the presence of 100 μM BAPN, alone (lane 5), nor the presence of 10 nM LO in the absence of the addition of BAPN or bFGF (lane 6) significantly affected the state of phosphorylation of MAP kinases in these growth arrested cells. As expected, MAP kinase activation by 0.28 nM bFGF was also inhibited by 200 μM genistein, a MAP kinase inhibitor (lane 7). None of these treatments exerted significant effects on the protein levels of MAP kinases during the experimental period (Fig. 4B). These results are consistent with the suppressive effect of LO on the increase in cell cycle progression elicited by bFGF.

Inhibition of bFGF Nuclear Localization by LO

Extracellular bFGF can be taken up into cells by endocytosis and then enter the nucleus within a variety of cell types in a cell-cycle-dependent manner [Prats et al., 1989; Baldin et al., 1990; Stachowiak et al., 1996; Arese et al., 1999; Nugent and Iozzo, 2000]. Thus, we investigated the possibility that oxidation of bFGF by LO may affect the nuclear localization of this growth factor into the Swiss 3T3 (control) fibroblasts. Fluorescence microscopy of cell probed with monoclonal anti-bFGF as primary antibody followed by incubation with FITC-conjugated rabbit anti-mouse IgG revealed a relatively minor degree of endogenous bFGF within the nuclei of growth-arrested Swiss 3T3 cells, while significantly greater amounts were seen in the cytoplasm (Fig. 5A). Incubation of cells with 0.28 nM bFGF for 1 h significantly increased immunodetectable bFGF within nuclei, as evidenced by brightly stained nucleolar clusters (Fig. 5B; see arrowhead), consistent with results previously described [Arese et al., 1999]. In contrast, prominent bFGF staining was observed only in the perinuclear region in cells which had been simultaneously incubated with bFGF and 10 nM LO, while intranuclear bFGF content was not enhanced in these cells (Fig. 5C). The inhibition of nuclear translocation of bFGF by LO was prevented by inclusion of 100 μM BAPN in the incubation medium together with LO and bFGF. Under these circumstances, bFGF prominently accumulated within the nucleus as evidenced by the appearance of brightly stained nucleoli (Fig. 5D). Importantly, inhibition of endogenous LO activity levels by incubation of cells with BAPN in the absence of added LO increased bFGF transport to the nucleus (Fig. 5E; see arrowhead). Addition of LO in the absence of exogenous bFGF or BAPN reduced endogenous levels of nuclear bFGF (Fig. 5F). These results indicate that the oxidation of bFGF by endogenous or exogenous LO inhibits the nuclear localization of bFGF, thus pointing to a mechanism for the LO-mediated inhibition of bFGF-stimulated cell cycle progression [Bikfalvi et al., 1995; Stachowiak et al., 1996; Arese et al., 1999].

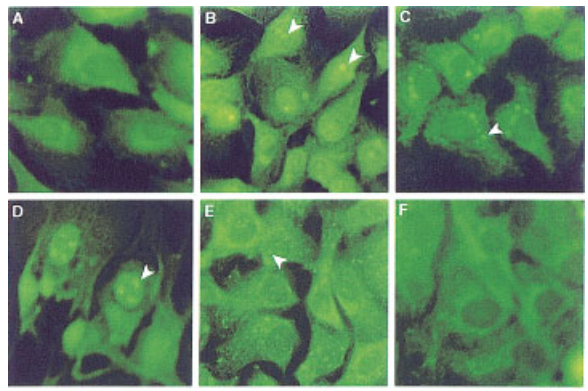


Fig. 5. Inhibition by LO of nuclear localization of bFGF in bFGF-stimulated Swiss 3T3 cells. Photomicrographs are at identical magnifications. Control cells (**A**); cells treated with 5 ng/ml bFGF (**B**); or with 5 ng/ml bFGF + 10 nM LO (**C**); or with 5 ng/ml bFGF + 10 nM LO + 100 μM BAPN (**D**); or with 100 μM BAPN alone (**E**); or with 10 nM LO, alone (**F**). Omission of the primary antibody resulted in the absence of staining (not shown).

Growth Suppression by LO of bFGF-Dependent Transformed Cells

To further evaluate the antiproliferative property of LO, we assessed the effects of exogenously added enzyme on the growth of 6-1 cells [Rogelj et al., 1988; Yayon and Klagsbrun,

1990]. These cells are highly transformed due to the expression of excess bFGF thus activating an internal autocrine, growth-stimulating mechanism [Yayon and Klagsbrun, 1990]. Indeed, injection of 6-1 cells into syngeneic NIH/NSF mice induced rapidly growing tumors within one week [Rogelj et al., 1988]. Serum-starvation culture conditions were chosen to assess relative growth rates of the parental NIH 3T3 cells and the 6-1 cells. Each cell line was initially plated at 2×10^4 cells in 35-mm dishes containing 2 ml 0.3% FBS/DMEM. The 6-1 cells increased several-fold in number during 5 days in these culture conditions, consistent with their transformed phenotype. In contrast, all of the parental NIH 3T3 cells lost viability within the same period (data not shown). These results appeared to validate the use of 0.3% FBS/DMEM to assess the effects of LO on the growth of these transformed cells. As shown in Figure 6A), exposure of 6-1 cells to nanomolar concentrations of LO for 6 days in 0.3% FBS/DMEM resulted in the dose-dependent inhibition of cell growth. Full inhibition was seen at 12.5 nM LO which decreased cell numbers by 81% in comparison to the control which was not incubated with added LO. The LO-mediated inhibition of the growth of these transformed cells was abolished by inclusion of 100 μM BAPN in the LO-supplemented medium. It is of further interest that the proliferation of the 6-1 cells which had been incubated in the presence of both BAPN and LO exceeded by 2.3-fold that of the control cells which had been incubated in the absence of both the LO and BAPN additives (Fig. 6B). Moreover, the inclusion of BAPN in the culture medium in the absence of added LO also markedly enhanced the growth of these cells (2.7-fold of the control; Fig. 6B), indicating that the catalytic activity of endogenous LO in these cells limits the stimulation of cell proliferation due to endogenous, excess production of bFGF. Since addition of exogenous LO in the absence of BAPN reduced basal proliferation by approximately 80% (Fig. 6B), the ambient level of the endogenous enzyme appears to be the insufficient to fully suppress proliferation. Direct assays of medium conditioned by the 3T3 6-1 cells for 16 h in 0.3% serum revealed the presence of detectable levels of LO enzyme activity (2,798 ± 562 cpm ^3H_2O per hour per 10^6 cells) assayed against a tritiated tropoelastin substrate [Bedell-Hogan et al., 1993]. Comparing this level of activity to that of pure

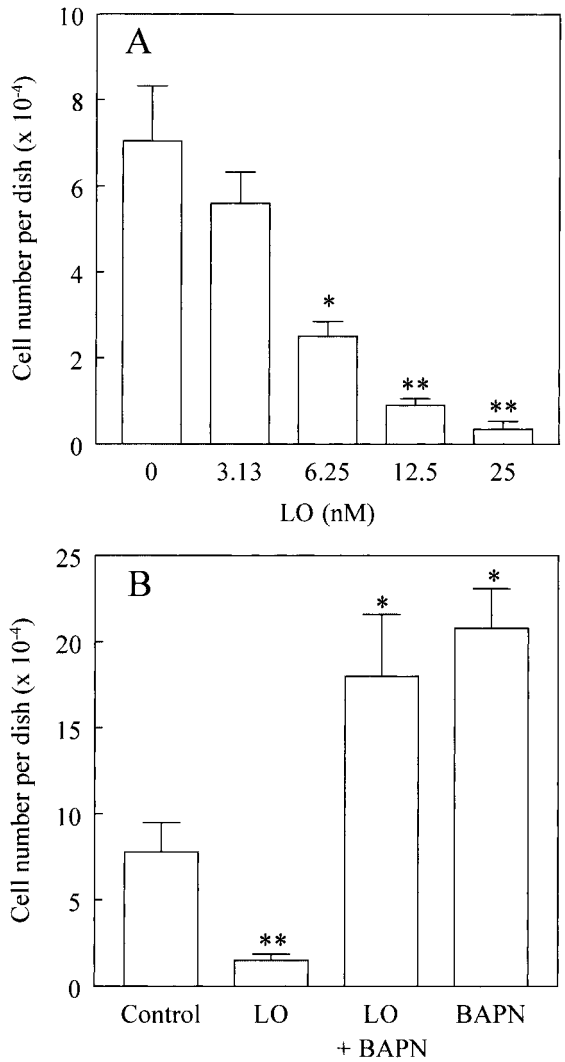


Fig. 6. Inhibition of bFGF-dependent transformed cell growth by LO. Transformed 6-1 cells (2×10^4) overexpressing bFGF were plated and incubated overnight in 35-mm dishes containing 2 ml 10% FBS/DMEM and then refed with fresh 0.3% FBS/DMEM with different treatment regimens as shown in (**A**) and (**B**). **A**: Dose response. Media was supplemented with LO at the indicated concentrations and cells were incubated for additional 6-day periods. Cultures were refed with fresh media supplemented with LO, as indicated, every 2 days. Cells were harvested by trypsinization and counted with a hemacytometer. **B**: Effects of inactivation of LO by BAPN on tumor cell growth. LO was present at 12.5 nM and BAPN at 100 μM as indicated. Data represent the means (±SD) of three separate experiments each assessed with triplicate dishes. Asterisks indicate values significantly different from the control as determined by ANOVA analysis: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

bovine aorta LO permits the estimation of the concentration of LO secreted by these cells as 2–3 nM. This endogenous enzyme activity was fully inhibitable by inclusion of 100 μM BAPN in the assay mixtures. As shown, nearly maximal

inhibition of the bFGF-stimulated growth of these cells is achieved by the increase in LO available to the cells by the addition of exogenous LO at 10–12 nM (Fig. 6A).

Examination by fluorescence microscopy of 6-1 cells which had been incubated with FITC-conjugated LO revealed that this fluorescently-labeled LO species was internalized by these cells in a time-dependent manner and localized within the nuclei (Fig. 7A,B), consistent with prior evidence that extracellular LO is readily transported into intracellular compartments [Nellaiappan et al., 2000]. Figure 7C shows the distribution of endogenous LO in 6-1 cells visualized by indirect immunofluorescence staining again indicating localization of LO in the cytoplasm and within the nuclei of these cells.

The possibility that exposure of the cells to LO alters the physical state of the bFGF produced by the 6-1 cells was assessed by Western blotting with monoclonal anti-bFGF of cell lysates. As shown in Figure 8, comparison of the bFGF content of NIH 3T3 cells (lane 1) and transformed 6-1 cells (lane 2) reveals significantly increased levels of bFGF in the 6-1 cells in

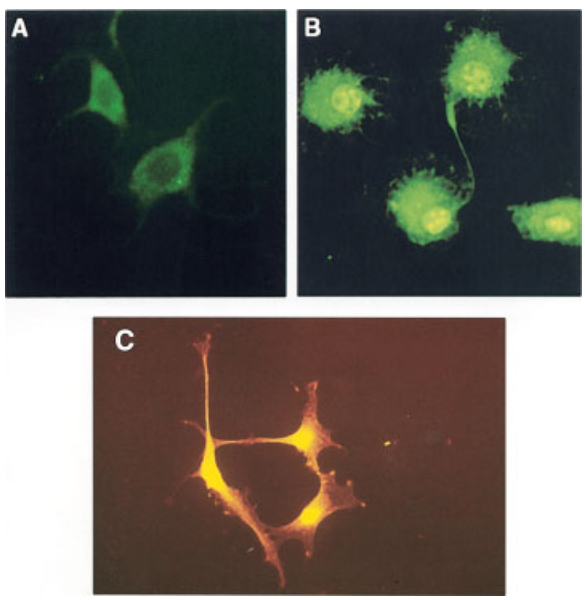


Fig. 7. Exogenous LO uptake by and endogenous LO distribution in 6-1 cells. Cells grown on coverslips were exposed to FITC-conjugated LO (0.16 μg/ml) for 15 min (**A**) and 4 h (**B**), respectively, then fixed and examined by fluorescence microscopy (100×). Endogenous LO in cells was revealed by indirectly immunofluorescent staining using the primary polyclonal anti-LO antibody and the second antibody conjugated with rhodamine (**C**).

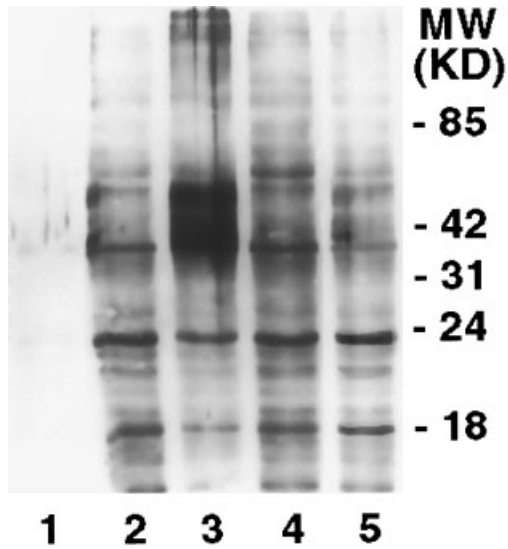


Fig. 8. Crosslinking of intracellular bFGF by LO. Subconfluent NIH 3T3 and 6-1 cells were incubated for 20 h in 0.3% FBS/DMEM with or without additives as indicated below. Cells were suspended by trypsin treatment, lysed, and processed as described in the text. Aliquots containing 50 μg of cell protein were analyzed by Western blotting of SDS–PAGE electrophoretograms probing with monoclonal anti-bFGF. bFGF expression in NIH 3T3 cells (**lane 1**); in 6-1 cells incubated in the absence of LO (**lane 2**); in 6-1 cells incubated in the presence of 12.5 nM LO (**lane 3**) or in the presence of 12.5 nM LO which had been preincubated with 100 μM BAPN for 30 min at 37°C (**lane 4**) or in the presence of BAPN, alone (**lane 5**).

comparison to the negligible quantities of bFGF found in the control NIH 3T3 cells (lane 1), as expected. As shown (lane 2), prominent bFGF bands derived from the 6-1 cells occur at 18 and 24 kDa with lesser bands seen at the 35–50 kDa range. The bands at 18–24 kDa are consistent with the expected sizes of the 18–24 kDa monomers of bFGF [Bikfalvi et al., 1995; Stachowiak et al., 1996; Arese et al., 1999]. This distribution of bFGF forms in the 6-1 cells is also consistent with the distribution of ectopic bFGF forms produced by these transfected cells in the initial descriptions of this cell line [Rogelj et al., 1988, 1989]. These authors excluded the retention of the signal peptide as a basis of the larger (>18 kDa) forms and suggested that the additional forms might reasonably result from post-translational modification of the bFGF during its passage through the endoplasmic reticulum and Golgi [Rogelj et al., 1988]. These authors also noted that the ectopically produced bFGF remains associated with these cells and is not secreted. In preparation for lysis, we had

incubated the intact cells with trypsin/EDTA, thus increasing the likelihood that the bFGF found in the cell lysate used for the Western blot analysis derived from the intracellular compartment. The levels of the 18 and 24 kDa bFGF bands are clearly reduced in extracts of the 6-1 cells which had been cultured in the presence of exogenous LO, while markedly increased levels of bFGF forms occur at the regions corresponding to 35–50 kDa (Fig. 8, lane 3). The distribution and densities of bFGF bands in extracts of 6-1 cells incubated with exogenous LO in the presence of BAPN are essentially the same as those seen in the extract of cells incubated in the absence of exogenous LO (Fig. 8, lane 4). Thus, these data reveal that the addition of LO results in the conversion of monomeric forms of intracellular bFGF to higher molecular weight forms whose molecular masses are consistent with crosslinked polymeric forms of the 18 and 24 kDa monomers. Examination of the profiles of the total protein in Coomassie blue stained electrophoretograms of the 6-1 cell lysates did not reveal evident changes in the distribution of the total protein bands between the control and LO treated cells (data not shown), indicating that the enzyme did not oxidize proteins indiscriminately in these cells.

DISCUSSION

The present studies reveal that bFGF, a key mitogen for normal and tumor cell growth, is a substrate of LO, the enzyme that initiates intra- and intermolecular crosslinking within fibrous collagen and elastin in the ECM. The oxidation of lysine in bFGF resulted in the loss of much of its potential to activate ERK1/2 MAP kinases, to stimulate cell cycle progression and to be taken up into the nuclei of Swiss 3T3 cells. Moreover, incubation of LO with 6-1 cells strongly inhibited the growth of these tumorigenic cells. Under the culture conditions used here to discrimate bFGF-dependent stimulation of the proliferation of these cells and in view of the demonstrated susceptibility of the bFGF of the 6-1 cells to LO-catalyzed oxidation, it seems likely that the inhibition of the bFGF-dependent proliferation of the 6-1 cells by LO reflects the destruction of the mitogenic effect of the cell-associated bFGF by LO. Thus, these results illustrate a mechanism whereby LO may function as a suppressor of normal and excessive rates of cell proliferation by virtue of its enzymatic modification of this growth factor.

The fluorometric assays of the oxidation of the 18 kDa bFGF species revealed that 5–6 of the 14 lysine residues in the 155 amino acid sequence of this growth factor were oxidized by LO at 37°C, whereas apparently all 14 lysines within the molecule were oxidized at 55°C. Lag phases were observed at the onset of the enzymatic reaction at both temperatures, consistent with the requirement for thermally-induced conformational changes exposing a limited number of lysines at the physiological temperature and all of the lysines at the higher temperature. Continuing studies will assess the possibility that, at physiological temperature, peptidyl lysine substrates of LO may include lysines 26, 125, and/or 135, known to be involved in the binding of bFGF to HSPG, and/or the lysine 110 component of the tyrosine kinase receptor domain within bFGF [Eriksson et al., 1991; Faham et al., 1996], all of which are likely to be among the more accessible lysines within bFGF.

Other possible peptidyl lysine substrates of LO within bFGF could include lysines 21, 52, 66, 77, 129, and/or 135. Thus, bFGF monomers have a high tendency to form non-covalent dimers or oligomers with these 6 lysine residues occurring at the interface of two interacting molecules [Venkataraman et al., 1996]. Once spontaneous self association has occurred and the lysines at the surface and/or interface of molecules are oxidized by LO, the presence of lysine-derived α-aminoadipic-δ-semaldehyde residues could then result in spontaneous and covalent, intermolecular crosslink formation, consistent with the present observations that polymers of bFGF are formed following incubation of the growth factor with LO. Clearly, lysines play critical roles in the expression of the biological functions of bFGF. The susceptibility of bFGF to oxidation by LO is consistent with earlier studies revealing that the expression of LO activity in vitro is favored by an electrostatic relationship between this acidic catalyst and proteins that are basic in character [Kagan et al., 1984].

MAP kinases are well-characterized intracellular mediators of cell proliferation which are activated by various growth factors including bFGF [Maher, 1999]. We have shown here that incubation of Swiss 3T3 cells with bFGF enhanced levels of phosphorylated forms of extracellular signal-regulated kinase 1/2 (ERK1/2)

i.e., mitogen-activated protein kinases (MAPK). This activation was markedly inhibited in the presence of LO. The inhibition by LO was prevented by inclusion in the medium of BAPN, an active site inhibitor of LO. The cell cycle progression from the G_0 and G_1 to the S and G_2/M phases which was stimulated in these cells by bFGF was markedly suppressed by LO in a manner which also required expression of the catalytic function of this enzyme. As noted, expression of the full biological effects of bFGF requires its interaction with both HSPG receptors and tyrosine kinase receptors on the cell surface and these interactions are dependent upon specific lysine residues at these interacting sites within the growth factor. The inhibiting effect of LO on the activation of MAPK and the induction of cell cycle progression likely reflect the oxidation of such lysine residues. Since oxidation by LO alters cationic lysine residues to non-ionic aldehyde functions, the present results provide a novel, enzymatic approach to illustrate and/or block the critical roles of ε-amino groups of bFGF in the biological functions of this growth factor previously noted.

The accumulation of bFGF in the perinuclear region of Swiss 3T3 cells incubated in the presence of LO suggests that transport of intracellular bFGF into the nucleus was specifically disrupted. Although both lysine-rich and arginine-rich sequences constitute consensus nuclear localization sequences of many nuclear proteins and the LO-catalyzed modification of bFGF would result in modified lysine residues, the nuclear import of bFGF appears to involve lysine-free, arginine-rich sequence regions [Pintucci et al., 1996; Arese et al., 1999]. Spontaneous formation of covalent crosslinks within or between bFGF molecules could underlie the decreased nuclear localization by altering the accessibility of the arginine-rich sequences. It is also possible that the H_2O_2 product of bFGF oxidation by LO may contribute to the suppression of the nuclear translocation of bFGF [Czubbryt et al., 2000].

The marked inhibition of the growth of 6-1 cells by inclusion of LO in the growth medium can be reasonably attributed to the oxidation by LO of the bFGF endogenously produced by these transformed cells. Since LO readily enters the intracellular compartment and in view of the apparent intracellular retention of bFGF by the 6-1 cells [Rogelj et al., 1988; Yayon and Klagsbrun, 1990], it appears most likely that the oxidation of bFGF by LO occurs intracellularly in these 6-1 cells. Of further interest, the stimulation of 6-1 cell proliferation by BAPN in the absence of added LO infers that endogenous LO partially suppresses the basal levels of 6-1 cell proliferation. This may well reflect the ability of endogenous enzyme to oxidize the endogenous growth factor, although other growth stimulating protein factors within or available to these cells may also be regulated by this catalyst. Consistent with this possibility, the response of platelet-derived growth factor (PDGF) and insulin-like growth factor 1 (IGF-1) receptors to their ligands is impaired by transfection of transformed cells with anti-sense LO cDNA [Giampuzzi et al., 2001].

Under physiological conditions, the balance between growth factors and growth inhibitors is critical to the maintenance of homeostasis of tissues and organs. The present data have demonstrated that bFGF, a mitogen acting on cells of mesodermal, neuroectodermal as well as other endodermal origins [Rifkin and Moscatelli, 1989; Nugent and Iozzo, 2000] is a substrate of LO. As a consequence of its oxidation by LO, both the mitogenic activity and nuclear localization of bFGF are significantly reduced. There is a growing body of evidence that LO can exert an anti-proliferative effect on cells. Thus, the mouse *ras* recission gene, *rrg*, the product of which suppresses Ha-ras-induced tumorigenesis, shares 92% DNA sequence homology and 96% identity at the amino acid sequence level with mouse LO [Contente et al., 1990; Kenyon et al., 1991]. Moreover, microinjection of recombinant LO blocks the effects of both oncogenic p21-H-*ras* and progesterone on the maturation of *Xenopus laevis* oocyte [DiDonato et al., 1997]. Consistent with its anti-oncogenic potential, LO is strongly down regulated in several tumorigenic and transformed cell lines [Kuivaniemi et al., 1986; Kenyon et al., 1991]. The widening distribution of possible LO substrates, now shown to include bFGF, raises important new possibilities regarding the roles that LO may play in biology above and beyond its function in stabilizing the macromolecules of the ECM.

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