Transformation of Epithelial Cells Stably Transfected with H2O2-generating Peroxisomal Urate Oxidase

Ruiyun Chu, Yulian Lin, Kirthi C. Reddy, Jie Pan, M. Sambasiva Rao, Janardan K. Reddy, and Anjana V. Yeldandi

Department of Pathology, Northwestern University Medical School, Chicago, Illinois 60611

Abstract

Peroxisome proliferators, a group of structurally diverse nongenotoxic agents, induce predictable pleiotropic responses in liver, including the development of liver tumors in rats and mice. These agents transcriptionally activate the three genes of the peroxisomal \( \beta \)-oxidation enzyme system by interacting with the peroxisome proliferator-activated receptor(s). It has been proposed that \( \text{H}_2\text{O}_2 \) generated by the peroxisomal \( \beta \)-oxidation system leads to DNA damage and neoplastic transformation. Consistent with this hypothesis is that cells stably transfected with \( \text{H}_2\text{O}_2 \)-generating peroxisomal fatty acyl-CoA oxidase cDNA, which encodes the first and rate-limiting enzyme of the \( \beta \)-oxidation system, undergo transformation in the presence of a fatty acid substrate. To test whether \( \text{H}_2\text{O}_2 \) generated by other peroxisomal oxidases can also lead to transformation, a full-length cDNA encoding rat urate oxidase (UOX), which oxidizes uric acid to allantoin and in the process generates \( \text{H}_2\text{O}_2 \), was introduced into African green monkey kidney cells (CV-1 cells) under the control of constitutively active human peroxisomal fatty acyl-CoA oxidase gene promoter. Five stably transfected CV-1 cell lines expressing recombinant rat UOX were isolated in which the recombinant protein was targeted to peroxisomes and formed crystallloid structures or cores similar to those present in rat liver peroxisomes. Increased levels of \( \text{H}_2\text{O}_2 \) were found when cells stably expressing UOX were exposed to the substrate uric acid. These five clones, designated A-U1 to A-U5, exhibited anchorage-independent growth, as demonstrated by the formation of transformed colonies in soft agar in proportion to the duration of exposure to uric acid. These transformants exhibited clonal growth under serum-deprived conditions. One of these transformed cell lines, the A-U3 cell line, was evaluated for tumorigenicity by s.c. injection in nude mice. All five mice injected with transformed A-U3 cells developed adenocarcinomas, but no tumors developed in mice injected with control CV-1 cells or cells stably expressing UOX that were not exposed to uric acid. These results provide further evidence indicating that sustained overexpression of a peroxisomal \( \text{H}_2\text{O}_2 \)-generating oxidase causes cell transformation.

Introduction

Peroxisomes are cellular organelles present in virtually all eukaryotic cells. These organelles contain more than 50 proteins and participate in many important metabolic processes (1-3). Of particular interest is that peroxisomes, present in mammalian liver, host at least five oxidases, namely ACOX, \( \text{UOX} \), \( \text{d}-\text{amino acid oxidase} \), \( \text{l}-\alpha \) hydroxy acid oxidase, and polyamine oxidase, which reduce \( \text{O}_2 \) to \( \text{H}_2\text{O}_2 \). The \( \text{H}_2\text{O}_2 \) generated within the peroxisome matrix is degraded by peroxisomal marker enzyme catale (1). Peroxisomal oxidases catalyze rapid \( \text{O}_2 \) uptake in the presence of physiological concentrations of substrates, and it has been estimated that these oxidases account for \( \sim 20\% \) of \( \text{O}_2 \) consumed in the liver (2). Among the peroxisomal oxidases, ACOX, the first and rate-limiting enzyme of the peroxisomal \( \beta \)-oxidation system (4, 5), has attracted considerable attention because of its remarkable inducibility and its postulated role in oxidative stress, leading to the development of liver tumors in rats and mice by peroxisome proliferators (6, 7). Peroxisome proliferators encompass a diverse group of synthetic and naturally occurring compounds, namely hypolipidemic drugs, phthalate ester plasticizers, industrial solvents, herbicides, hormones, and others (7). These agents induce qualitatively predictable pleiotropic responses in rats and mice, consisting of hepatomegaly, proliferation of peroxisomes in liver parenchymal cells, and the induction of several hepatic enzymes, particularly those of the peroxisomal fatty acid \( \beta \)-oxidation system (7). Continued exposure to peroxisome proliferators leads to the development of hepatocellular carcinomas in rodents, a delayed response attributed to sustained induction of peroxisome proliferation (7). It is now well established that peroxisome proliferation is associated with \( \sim 20-30 \)-fold increases in the activities of all three enzymes of the \( \text{H}_2\text{O}_2 \)-generating \( \beta \)-oxidation system, namely ACOX, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase, and 3-keetoacyl-CoA thiolase, but catalase, which degrades \( \text{H}_2\text{O}_2 \), increases less than 2-fold (8). The hepatocarcinogenicity of peroxisome proliferators has been postulated to be the result of prolonged oxidative stress in the liver imposed by the disproportionately increases in \( \text{H}_2\text{O}_2 \)-generating \( \beta \)-oxidation system enzymes and catalase, which degrades \( \text{H}_2\text{O}_2 \) (7, 8).

The available experimental evidence accumulated in favor of the oxidative stress hypothesis in peroxisome proliferator-induced hepatocarcinogenesis, as reviewed elsewhere (7, 9), includes: (a) marked increases in the receptor-mediated transcriptional activation of \( \text{H}_2\text{O}_2 \)-generating \( \beta \)-oxidation enzyme system genes; (b) minimal increases in the level of catalase, an enzyme that degrades catalase; (c) decreased levels of free radical-scavenging enzymes; (d) increased levels of \( \text{H}_2\text{O}_2 \), hydroxyl radicals, and 8-hydroxydeoxyguanosine lesions in the liver of rats chronically exposed to peroxisome proliferators; (e) endogenous DNA alterations detected by the \( ^{32}\text{P} \) postlabeling assay; and (f) accumulation of lipofuscin in liver parenchymal cells, a morphological parameter of oxidative damage. Recently, we have provided more direct evidence by demonstrating that stable overexpression of rat peroxisomal ACOX is sufficient to transform epithelial cells in the presence of a fatty acid substrate (10). We now hypothesize that overexpression of other \( \text{H}_2\text{O}_2 \)-generating oxidases, in particular, peroxisomal oxidases such as UOX, should also lead to neoplastic transformation under appropriate in vivo and in vitro conditions. Identification of \( \text{H}_2\text{O}_2 \) as the proximate cause of transformation, irrespective of the nature of the oxidase that generates this oxidant, is essential for further studies on the mechanism by which \( \text{H}_2\text{O}_2 \) mediates transformation. To accomplish this goal, we have stably transfected CV-1 cells with rat UOX cDNA driven by the human peroxisomal ACOX gene promoter. UOX, present as a crystallloid core within the liver peroxisome, degrades uric acid to allan-
toin and in the process generates H$_2$O$_2$ (1). We show that CV-1 cells overexpressing UOX undergo transformation when exposed to the substrate uric acid, and such transformed cells form tumors when injected into nude mice. These observations provide further support for the role of H$_2$O$_2$ in neoplastic transformation and, by inference, for its role in peroxisome proliferator-induced liver carcinogenesis.

Materials and Methods

**Plasmid Construction.** The plasmid pHACOX was constructed by cloning the most upstream 3.7-kb ScaI-BamHI promoter fragment of the human ACOX gene from the plasmid pSKACOX (11) with the 294bp of the minimal tomo and in the process generates H$_2$O$_2$ (1). We show that CV-l cells overexpressing UOX undergo transformation when exposed to the cultured in the presence of G418 (Life Technologies, Inc.) for up to 3 weeks. CV-l cells were cotransfected with 10 μg pHACOXUOX DNA and 1 μg pL plasmid DNA, and then the rat UOX cDNA (1.3 kb) was inserted into the blunt-ended NotI site. The construct in which the rat UOX cDNA is under the control of human ACOX gene promoter is designated pHACOXUOX.

**Cell Culture and Transfection.** CV-1 cells were cultured in MEM with 10% FCS, 2 mm glutamine, 1% fungizone, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified incubator in a 5% CO$_2$-95% air atmosphere. CV-1 cells were passaged by trypsin treatment once a week. Cells were plated at 5 × 10$^4$ cells/dish on 100-mm tissue culture dishes and cotransfected with 10 μg pHACOXUOX DNA and 1 μg plasmid DNA, which contains the gene encoding neomycin resistance, using the standard calcium phosphate precipitation method as described elsewhere (10). The cells were incubated for 24–48 h and then passaged into five 100-mm dishes and cultured in the presence of G418 (Life Technologies, Inc.) for up to 3 weeks. Individual G418-resistant colonies were obtained by the ring isolation technique and expanded separately.

**Southern and Northern Analysis.** Approximately 10 μg genomic DNA, prepared from cells and tumors, were digested with Xhol and HindIII at 37°C overnight and subjected to 1% agarose gel electrophoresis with 50 mM Tris-boric acid buffer (pH 9.0). Uric acid was added to the culture medium to a concentration of 250 μM (12), and the culture medium was changed once every 2 days. For colony formation in soft agar, a 0.5% agarose solution made with 250 μM Uric acid was added to the culture medium and overlayed on the previously formed agarose gels (4 ml containing 10$^3$ cells/dish). Plates were incubated at 37°C in 5% CO$_2$-95% air for 2–6 weeks. Phase-contrast micrographs were taken for evaluation, and the number of colonies with diameters of >0.2 mm was counted.

**Tumorigenility.** Male nu/nu mice (Harlan Sprague-Dawley) were used to ascertain the tumorigenicity of transformed cells (10). Briefly, colonies grown in soft agar were recultured in MEM, trypsinized, and suspended in PBS. Approximately, 5 × 10$^6$ cells in 0.2 ml PBS were injected s.c. into nude mice. Control animals were injected with either CV-1 cells or untreated transfectants. Histological examination was performed on tumors removed when they attained 0.4–1 cm.

**Other Methods.** Uric acid levels in culture medium were determined spectrophotometrically by monitoring the absorption at 292 nm (12). H$_2$O$_2$ production was measured by the phenol red method (13). Primers used for the detection of the UOX transgene by PCR amplification were: sense, 5'-GGG AAA ATG GCC CAT TAC CAT GAC-3'; and antisense, 5'-ATA ACG TCA CAG CCT GGA AGG CAG-3'.

**Results**

**Characterization of pHACOXUOX Transfectants.** CV-1 cells were cotransfected with an expression vector, pHACOXUOX, which has the rat UOX cDNA under the control of the human ACOX promoter (Fig. 1A) and a neo$^R$ plasmid and then cultured in the presence of G418. After 3 weeks of culture, five neomycin-resistant colonies were obtained. Five independent colonies were characterized by Southern and Northern hybridization. All five neomycin-resistant colonies showed integration of the rat UOX cDNA, as determined by Southern hybridization (Fig. 1B) and PCR analysis (data not shown). These positive transfectants were designated A-U1−A-U5. Northern blotting revealed that these five clones expressed UOX mRNA (Fig. 1C) and revealed UOX protein on immunoblotting (Fig. 1D). Among these transfectants, A-U4 displayed low-level expression of UOX mRNA and protein, whereas others showed similar levels of expression. No endogenous UOX mRNA or protein was detected in the untransfected CV-1 cells (Fig. 1, C and D).

**Targeting of Rat UOX Into Peroxisomes in CV-1 Cells.** When CV-1 cells stably expressing recombinant rat UOX (A-U1–A-U5) were subjected to immunofluorescence examination to visualize the distribution of UOX, much intensely fluorescent, discrete granular staining was found in the cytoplasm (Fig. 2A), whereas untransfected CV-1 cells showed no such staining (Fig. 2B). UOX appeared as fine granular staining in the cytoplasm of CV-1 cells stably expressing UOX when compared with the large, clumpy staining noted in *S. frugiperda* (Sf9) insect cells expressing rat UOX (Fig. 2C), using the baculovirus expression system (14). The absence of identifiable peroxisomes in Sf9 insect cells and the powerful baculovirus promoter appear to generate massive aggregates of recombinant UOX both in the cytoplasm and the nucleus of insect cells (14). In species that express UOX, this protein is identified as a nuclear core or crystalloid within the peroxisomes of liver parenchymal cells. Targeting of a majority of proteins to peroxisomes is directed by the consensus sequence of three amino acids, Ser-Lys-Leu, at the carboxyl terminus of the protein (15), and this tripeptide or a conserved variant, such as Ser-Arg-Leu present in rat UOX, appears sufficient for directing proteins to peroxisomes. Accordingly, the transmission electron microscopic observations confirmed that the recombinant rat UOX expressed in CV-1 cells is targeted to peroxisomes in these cells.
URATE OXIDASE-MEDIATED TRANSFORMATION

Fig. 1. Schematic diagram of ACOX-UOX construct and characterization pHACOXUOX transfectants. A, structure of pHACOXUOX construct. A 4-kb fragment of the human ACOX gene promoter (I) was cloned into the EcoRI and SacI site of a promoterless reporter plasmid, pNASSb. The reporter β-galactosidase-coding sequence was replaced by rat UOX cDNA. The resulting plasmid was designated pHACOXUOX. B, Southern analysis of DNA isolated from independent pHACOXUOX transfectants (A-U1–A-U5) and untransfected CV-1 cells. CV-1 cells cotransfected with the pHACOXUOX construct and a helper plasmid-carrying neomycin gene driven by the SV40 early promoter were selected by G418 over a 3-week period. G418-resistant colonies were isolated and expanded. DNA (10 μg) from each clone and the untransfected CV-1 cells was digested with XhoI and HindIII, separated on a 1% agarose gel, transferred to a nitrocellulose membrane, and probed with 32P-labeled UOX cDNA. C, Northern blot analysis for expression of UOX mRNA. Total RNA (10 μg) was hybridized with the UOX cDNA probe as in B. D, Western blot analysis for expression of UOX protein. Cellular proteins (75 μg) from each cell line were separated by 10% SDS-PAGE, transferred to a nitrocellulose membrane, and stained with a specific antibody against rat UOX. Lanes A-U1–A-U5, five independent clones; Lane CV-1, untransfected CV-1 cells as a control.

Transmission electron microscopic examination of CV-1 cells stably expressing UOX revealed electron dense structures and inclusions within single membrane-limited cytoplasmic organelles (Fig. 3, A–C). These organelles were identified as peroxisomes by demonstrating the presence of the peroxisomal marker enzyme catalase (12). No crystallloid core structures were detected in normal, untransfected CV-1 cells (Fig. 3D). These dense inclusions, seen exclusively in stable transfectants expressing recombinant UOX, are composed of bundles of hollow tubules, similar to those present in rat hepatic peroxisome cores (12). UOX has been localized in these hollow tubular cores by immunogold staining (data not shown). Thus, the rat UOX cDNA driven by the human ACOX promoter is successfully expressed and properly targeted to peroxisomes in CV-1 cells.

Uric Acid Metabolism and Production of H2O2 by pHACOX-UOX Transfectants. Mammals other than primates oxidize uric acid to allantoin as their excretory product (16). The reaction catalyzed by

Fig. 2. Immunofluorescence staining of cells stably transfected with rat UOX cDNA and untransfected CV1 cells. Cells were grown on coverslips and fixed with cold methanol. The fixed cells were treated with 0.5% Triton X-100 for 5 min and blocked with 5% BSA for 1 h. A rabbit polyclonal antibody raised against rat UOX was used as a primary antibody and a monoclonal antirabbit IgG antibody conjugated with tetramethylrhodamine isothiocyanate was used as secondary antibody to stain the cells. Note that the punctuate granules are extensively located in the cytoplasm of the cells stably transfected with pHACOXUOX (A). No stain is seen in control CV-1 cells (B). In SF9 insect cells expressing recombinant rat UOX, the larger granules are present in the nucleus and cytoplasm (C). These cells were stained with the same primary antibody and a secondary antibody conjugated with fluorescent isothiocyanate.
UOX uses \( \text{O}_2 \) as the final electron acceptor, which is further converted to \( \text{H}_2\text{O}_2 \). As we showed earlier, CV-1 cells expressing UOX, under the control of a cytomegalovirus promoter, can convert uric acid into allantoin \textit{in vitro} (12). We examined the ability of cells stably transfected with pHACOXUOX to consume uric acid and to produce \( \text{H}_2\text{O}_2 \). Uric acid was added to the culture medium to a final concentration of 250 \( \mu \text{M} \). After 24 h, the concentration of uric acid that remained in the culture medium was measured (Fig. 4). All five cell lines expressing UOX under the control of the human ACOX promoter reduced uric acid concentration in the medium by about 100 \( \mu \text{M} \) within 24 h, whereas the cellular \( \text{H}_2\text{O}_2 \) level increased to \( \sim 20-30 \mu \text{M} \) (Fig. 4). In contrast, untransfected CV-1 cells were unable to convert uric acid into allantoin, and these cells exhibited low levels of cellular \( \text{H}_2\text{O}_2 \) (Fig. 4).

**pHACOXUOX Transfectants Exhibit Anchorage-independent Growth following Exposure to Uric Acid.** To examine whether cells overexpressing UOX can undergo transformation when treated with the substrate uric acid, we exposed all five transfectants (A-U1—A-U5) to uric acid for up to 60 days. All five transfectants when exposed to uric acid for 60 days revealed disorganized growth characteristics and increased cell density. In particular, the A-U3 cell line, which has the highest UOX expression, began to show indications of transformation as early as 15 days after exposure to uric acid. At confluence, the transfectants grew to a significantly higher cell density due to their underlapping and overlapping growth characteristics (Fig. 5B). These cells exhibited an unorganized growth pattern, with the formation of heaped-up refractile aggregates characteristic of transformed cells, whereas CV-1 and untreated cells showed a typical monolayer appearance (Fig. 5A). When cultured within agarose gels, these treated UOX-expressing cells remained viable, proliferated, and formed spherical colonies, indicating that they acquired anchorage-independent growth characteristics (Fig. 5, C and D). Transfectants untreated with uric acid, as well as untransfected CV-1 cells, when cultured within agarose gels failed to proliferate and died (data not shown). The number of colonies formed was dependent on the duration of exposure to uric acid (Fig. 6). When cells isolated from colonies from soft agar were recultured in dishes, they exhibited clonal growth under serum-deprived conditions (data not shown).

**Tumorigenicity in Nude Mice.** We examined the growth potential of the transformed A-U3 cell line by reculturing cells isolated from independent colonies formed in soft agar and then ascertained the tumorigenicity of these cells by injecting into nude mice. These cells formed solid tumors within 3–6 months (Fig. 7A). Histological examination of these tumors revealed a distinct adenocarcinomatous pattern (Fig. 7B). PCR amplification of the DNA confirmed the presence of the \( \text{UOX} \) transgene in the initial transfectant A-U3 cells, 60-day uric acid-treated A-U3 cells, colonies recovered from soft agar (transformed A-U3T cells), and tumors formed in nude mice (Fig. 8A). Nevertheless, the expression of UOX in these tumors was confirmed by Northern blot hybridization (data not shown) and by immunoblotting (Fig. 8B). No tumors developed when control CV-1 cells were exposed to uric acid for up to 60 days.
cells or A-U3 cells stably expressing UOX were injected into nude mice.

Discussion

Oxygen and nitrogen radicals are involved in a variety of normal cellular functions. These free radicals and hydroperoxides, referred to as ROS, are increasingly being implicated in a number of disease processes, and it is generally assumed that the normal level of the antioxidant defense system is not efficient for the eradication of ROS-induced deleterious effects (17). The resultant cumulative effect of molecular damage caused by oxidative stress is postulated to contribute to atherosclerosis, aging, and cancer (17). Studies from our laboratory have implicated oxidative stress caused by a sustained increase in ACOX activity as the basis for hepatocarcinogenesis induced by peroxisome proliferators (3, 8, 9). ACOX, the first gene of the peroxisomal β oxidation system that generates H2O2, as well as other peroxisome proliferator responsive genes are transcriptionally activated by peroxisome proliferators through a receptor-mediated mechanism (3, 18). Peroxisome proliferators activate members of the nuclear receptor superfamily, termed peroxisome proliferator-activated receptors, which play a pivotal role in the peroxisome proliferator-induced pleiotropic responses (3, 18). Genetic damage could ensue from H2O2 escaping out of the peroxisomal network and from possible resultant ROS in the livers of rats and mice with sustained induction of peroxisome proliferation and the H2O2-generating peroxisomal ACOX (7, 9).

In the present study, we used the human ACOX promoter to drive the rat UOX cDNA in CV-1 cells. The expression of UOX appeared similar to that observed when rat UOX was expressed under the control of the cytomegalovirus immediate-early gene promoter (12). We now show that CV-1 cells stably expressing functionally active rat UOX, which has been properly targeted to peroxisomes, undergo transformation when treated with the substrate uric acid for up to 60 days. UOX catalyzes the conversion of uric acid to allantoin using O2 and in the process generates H2O2. Our observations confirm marked increases in the levels of intracellular H2O2 in all of the transfectants in the presence of uric acid, and continued exposure to uric acid imparted to these cells the ability to grow in soft agar. These transformants, when recultured on a plastic surface under low serum concentrations, exhibited higher growth potential, and such cells formed tumors in nude mice. These results extend our previous observations with stable overexpression of peroxisomal ACOX in CV-1 cells, which when exposed to the substrate linoleic acid underwent neoplastic transformation (10).

The present study with UOX expression and our earlier results with ACOX expression in CV-1 cells clearly establish that overexpression of a H2O2-generating oxidase in CV-1 cells can trigger neoplastic transformation when such cells are exposed to the proper substrate. Overexpression of ACOX and UOX, both peroxisomal oxidases, results in increased cellular H2O2 levels when exposed to the appropriate substrate, but the mechanism by which increased H2O2 levels lead to epithelial cell transformation is unclear at present. It is conceivable that pHACOXUOX transfectants may have higher levels of DNA-damaging free radicals in response to uric acid substrate. Although data on the levels of antioxidant enzymes in these cells are not available, it is generally held that cells in culture exhibit reduced concentrations of such enzymes. We observed immunocytochemically detectable amounts of catalase in CV-1 cell peroxisomes (12), but
Fig. 7. Tumorigenesis of pHACOXUOX transfectants. A, colonies grown in soft agar were cultured, and 5 × 10⁶ cells were injected into nude mice. This photograph was taken 4 months after injection of cells. B, histological appearance of tumors that developed in nude mice. These slowly growing tumors display characteristic adenocarcinomatous differentiation.

detailed information on the levels of the antioxidant defense system in CV-1 cells and changes in the levels of these enzymes in these cells stably overexpressing UOX or ACOX is needed to evaluate the susceptibility of CV-1 cells to increased levels of ROS. In the livers of animals with peroxisome proliferation, a substantial reduction in the antioxidant capacity has been well documented, in addition to increases in the oxidant capacity (7–9, 19). Because the CV-1 cells contain zero endogenous UOX, the establishment of the pHACOX-UOX transfectants and the demonstration that overexpression of UOX can cause cell transformation provide a new model to study the biological role of the oxidants H₂O₂ and other ROS.

ROS induce the formation of 8-hydroxydeoxyguanosine adducts in nuclear DNA and participate in the process of mutagenicity, carcinogenicity, aging, and development of degenerative disease (17). It is pertinent to note that H₂O₂ and other reactive oxygen intermediates activate a variety of transcription factors, including nuclear factor κB, which plays a crucial role in the regulatory control of cells (20). ROS have been reported to induce single-strand breaks in cellular DNA, chromosomal aberrations, and DNA-protein cross-links (21). Furthermore, ROS are known to transcriptionally activate protooncogenes such as c-fos, c-myc, and c-jun (22). Evidence also points to the role of H₂O₂ and other ROS in activating the apoptosis programs in cells (23). The availability of cell lines overexpressing ACOX and UOX should enable us to investigate the mechanism by which increased intracellular H₂O₂ confers the characteristics of transformation.

References


Transformation of Epithelial Cells Stably Transfected with $\text{H}_2\text{O}_2$-generating Peroxisomal Urate Oxidase

Ruiyin Chu, Yulian Lin, Kirthi C. Reddy, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/56/21/4846

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.