Disruption of Redox Homeostasis in the Transforming Growth Factor-α/c-myc Transgenic Mouse Model of Accelerated Hepatocarcinogenesis*

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In previous studies we have demonstrated that transforming growth factor (TGF)-α/c-myc double transgenic mice exhibit an enhanced rate of cell proliferation, accumulate extensive DNA damage, and develop multiple liver tumors between 4 and 8 months of age. To clarify the biochemical events that may be responsible for the genotoxic and carcinogenic effects observed in this transgenic model, several parameters of redox homeostasis in the liver were examined prior to development of hepatic tumors. By 2 months of age, production of reactive oxygen species, determined by the peroxidation-sensitive fluorescent dye, 2',7'-dichlorofluorescin diacetate, was significantly elevated in TGF-α/c-myc transgenic hepatocytes versus either wild type or c-myc single transgenic cells, and occurred in parallel with an increase in lipid peroxidation. Concomitantly with a rise in oxidant levels, antioxidant defenses were decreased, including total glutathione content and the activity of glutathione peroxidase, whereas thioredoxin reductase activity and a very low activity of glutathione peroxidase. Furthermore, specific deletions were detected in mtDNA as early as 5 weeks of age in the transgenic mice. These data provide experimental evidence that co-expression of TGF-α and c-myc transgenes was severe DNA damage.

Current knowledge suggests that endogenous oxidants generated by multiple intracellular pathways may be considered as an important class of naturally occurring carcinogens (1, 2). Reactive oxygen species (ROS) are endogenous oxygen-containing molecules formed as normal products during aerobic metabolism (3). The term encompasses many species including superoxide (O$_2^-$), hydroxyl (HO·), peroxyl (RO$_2^-$), and alkoxyl (RO·) radicals, and certain nonradicals such as singlet oxygen (1O$_2$) and hydrogen peroxide (H$_2$O$_2$) that can be easily converted into radicals. ROS can produce genetic mutations as well as gross chromosomal alterations and thus contribute to cancer development at initiation, promotion and progression stages (4, 5). There is also accumulating evidence that oxidative damage to DNA may play a critical role in aging (6, 7).

In addition, a number of recent studies have demonstrated that ROS at submicromolar levels act as novel intra- and intercellular second messengers and thus modulate various aspects of cellular functions including proliferation, apoptosis and gene expression (8, 9). New evidence indicates that ligand binding to cell surface receptors linked to tyrosine kinase activity can trigger signal transduction pathways leading to intracellular ROS generation (10). An expanding list of extracellular stimuli shown to induce ROS generation in a variety of nonphagocytic cell types includes a number of peptide growth factors such as platelet-derived growth factor (11, 12), basic fibroblast growth factor (11–13), and epidermal growth factor (14, 15), as well as certain cytokines including tumor necrosis factor-α (13, 16, 17), interleukin-1 (16), and transforming growth factor (TGF)-β (18–24). Although the chemical nature of the ROS generated in response to the activation of various receptors has not been well characterized, H$_2$O$_2$ has been shown to be a major component of ROS in cells treated with EGF, platelet-derived growth factor, or TGF-β (12, 14, 15, 20, 22).

The stimulation of ROS production by TGF-α either in vitro or in vivo has not been yet reported. TGF-α is a member of a family of growth factors which elicit their growth regulation by triggering the EGF receptor signaling cascade (25). We have previously found that cooperation of TGF-α and c-myc pathways in the liver cells is extremely efficient in acceleration of hepatocarcinogenesis in double transgenic mice (26, 27). In fact, TGF-α/c-myc mice not only developed tumors more rapidly than either of the parental lines, but the multiplicity and tumor size were significantly increased, indicating escalation of both initiation and promotion stages of cancer development. Two hallmarks of hepatocarcinogenesis associated with TGF-α/c-myc signaling are widespread dysplasia (27) and profound chromosomal abnormalities (28). The rapid development of a dysplastic phenotype preceded the early onset of liver tumor growth and was associated with marked cellular enlargement, up-regulation of TGF-β, gene expression and growth cessation indicative of premature senescence (29, 30). However, the most remarkable biological consequence of constitutive co-expression of TGF-α and c-myc transgenes was severe DNA damage. In 2-month-old double transgenic hepatocytes, the frequency of chromosomal breakage was increased nearly 10-fold, whereas the number of aberrations observed in c-myc and TGF-α single transgenic mouse.

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‡ The abbreviations used are: ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorodihorofluorescin diacetate; EGF, epidermal growth factor; Gpx, glutathione peroxidase; 4-HNE, hydroxylalkenals; TGF, transforming growth factor; WT, wild type; bp, base pair(s); PCR, polymerase chain reaction; DCF, 2',7'-dichlorofluorescein.
transgenic hepatocytes was only 1.3- and 3-times background, respectively (28). Moreover, the presence of nonrandom chromosomal breaks recorded prior to tumor development and persistent enhancement of chromosomal damage in hepatocellular carcinomas \(^2\) suggested that TGF-α/c-myc hepatocytes exhibited a mutator phenotype (4). Given the known carcinogenic properties of ROS, we hypothesized that enhanced metabolic generation of oxygen radicals in rapidly proliferating TGF-α/c-myc transgenic hepatocytes might be responsible for genetic instability and acceleration of liver cancer in this animal model. The present study was undertaken to examine whether the chronic activation of mitogenic signaling induced by overexpression of c-myc and TGF-α transgenes creates a state of oxidative stress in mouse liver.

**EXPERIMENTAL PROCEDURES**

Materials—The following chemicals were used: collagenase (type H) (Boehringer Mannheim), and 2,7'-dichlorofluorescin diacetate (DCFH-DA) (Molecular Probes, Eugene, OR). GSH, glutathione assay kit, glutathione peroxidase assay kit, and lipid peroxidation kit were purchased from Bio-Rad, 10 mM EDTA, 10 mM 5,5'-dithiobis-(2-nitrobenzoic acid) method essentially as described previously (35). The tissue homogenates were prepared as above for GPx determinations and the assay mixtures (1 ml) contained 50 mM potassium phosphate, pH 7.0, 50 mM KCl, 10 mM NADPH, 0.2 mg/ml bovine serum albumin (0.2 mg/ml), and 2.5 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (50 μl of a 50 mM solution in absolute ethanol). The change in absorbance at 412 nm was monitored over 1 min at 30 °C. Activity was defined as micromoles of NADPH oxidized per min by ΔA412/(13.6 × 2), since 1 mol of NADPH yields 2 mol of thionitrobenzoate.

**Plasma Biochemistry**—Plasma was collected from retro-orbital puncture. The concentrations of alanine aminotransferase, triglycerides, and total cholesterol were measured utilizing an automated multichannel analyzer (Analytix Incorporated, Gaithersburg, MD).

**Other Analytical Methods**—Protein concentrations were determined using the Bio-Rad detergent-compatible protein assay with bovine serum albumin as the protein standard.

**Detection of mtDNA Deletion**—Total DNA was isolated from liver samples using the G NOME DNA isolation kit following the manufacturer’s instructions. Detection of mtDNA deletion present in the direct repeats of mtDNA (direct repeat 17 corresponding to bp 979–5650 of mouse mtDNA) (36) was performed by PCR utilizing the primers ATGCGAAACGGAAGGGAAC (bp 1094–1113) and ATGCTGAAGGAGGAA (bp 4915–4934) and the following conditions: denaturation (96 °C, 40 s); primer annealing (50 °C, 30 s); primer extension (72 °C, 2 min); 35 cycles. The PCR product of the intact repeat is 4671 bp, while the predicted size of the deletion between the direct repeats is 3821 bp, producing a PCR deletion product of 851 bp. The PCR products were analyzed on a 1% Tris-acetate-EDTA-agarose gel and visualized using ethidium bromide.

**Electron Microscopy**—Pieces of liver were fixed in a 100 mM phosphate-buffered solution (pH 7.4) of 2.5% glutaraldehyde and 2% neutral formaldehyde for 4 h and then postfixed in 100 mM phosphate-buffered 1% osmium tetroxide solution for an additional 2 h. After embedding in Epon 812, ultrathin sections were cut with a Diatome diamond knife on LKB ULTRATOM III ultratome (LKB Ultrotome, Uppsala, Sweden), then contrasted with uranyl acetate and lead citrate, and examined on a JEOL 100CX transmission electron microscope (Tokyo, Japan).

**Statistical Analysis**—Results are expressed as the mean ± S.E. The significance of the difference of means was determined by the paired Student’s t test.

**RESULTS**

**ROS Production Is Increased in Hepatocytes from TGF-α/c-myc Transgenic Mice**—To determine whether TGF-α/c-myc hepatocytes generate higher levels of ROS, DCFH-DA was utilized as a substrate for detection of H₂O₂ and other hydroperoxides (32, 37, 38). This assay involves the incorporation of the nonpolar compound, DCFH-DA, into the hydrophobic regions of the cell where it is hydrolyzed to DCFH. In the presence of appropriate oxidants, DCFH gives rise to DCF, which is membrane-impermeable and highly fluorescent, and can be detected by fluorescent-activated sorter analysis. Fig. 1A demonstrates that the rate of oxidation of DCFH-DA to DCF was comparable in 5-week-old WT, c-myc, and TGF-α/c-myc mice. However, by 10 weeks of age, double transgenic hepatocytes generated significantly greater amounts of peroxides, as shown by a shift to the right in the mean logarithmic fluorescence intensity to that observed in WT mice (Fig. 1B). Quantitative measurements showed that the levels of peroxide production was increased by 8.2-fold in TGF-α/c-myc transgenic hepatocytes versus 3.2-fold in c-myc cells between 5 and 10 weeks of age. No significant difference was found between the mean DCF fluorescence intensity in WT hepatocytes over a period of the first 2 months of life (Fig. 1A).

**Lipid Peroxidation Is Elevated in TGF-α/c-myc Livers**—We consider it a significant contribution to the understanding of the pathophysiology of chronic liver disease.
next examined the rate of lipid peroxidation as a downstream measure of accumulated oxidative damage to membrane lipids. Lipid peroxidation is important because it amplifies the free radical process, and because its products could lead to cellular and tissue damage (39). No differences were found in the tissue content of 4-HNE and malondialdehyde, the major aldehyde end products of membrane lipid peroxidation, in liver samples from WT and c-myc mice (Fig. 2). In contrast, approximately 2-fold higher levels of 4-HNE and malondialdehyde were present in TGF-α/c-myc livers compared with either age-matched WT and c-myc mice. In addition, double transgenic livers exhibited a greater sensitivity to collagenase perfusion and consistently yielded 3–5-fold less viable hepatocytes than age-matched WT controls, as judged by cell counting and trypan blue exclusion (data not shown), indicating altered membrane properties. Furthermore, by 10 weeks of age, c-myc and TGF-α/c-myc mice showed a 2- and 4-fold increase in the plasma levels of alanine aminotransferase, respectively (Fig. 3A), apparently as a consequence of cumulative free radical cytotoxic activity. In addition, double transgenic mice had higher plasma levels of cholesterol (Fig. 3B) and triglyceride (not shown). Histologically, TGF-α/c-myc hepatocytes displayed signs of “fatty liver” phenotype (Fig. 4) further suggesting a disorder in lipid metabolism. Together, these data demonstrate that co-expression of TGF-α and c-myc transgenes in mouse liver results in the creation of an oxidative stress environment and tissue damage starting at a very young age.
large 3821-bp mtDNA deletions associated with direct sequence repeats normally present in aging mice (36). Fig. 5 shows that this deletion was undetectable in 5- and 10-week-old WT livers in the absence of oxidative stress. In contrast, the deletion was readily detectable in both c-myc and TGF-a/c-myc mice as early as 5 weeks of age. The amount of deletion product was more extensive in the TGF-a/c-myc livers which produced higher levels of peroxides (Fig. 1). The presence of early mtDNA damage did not correlate with the loss of mitochondrial morphology. Electron microscopy showed no clear evidence of swelling or degeneration of mitochondria in randomly examined TGF-a/c-myc hepatocytes at 10 weeks of age. However, double transgenic hepatocytes from 10-week-old mice contained frequent residual bodies or lipofuscin granules and secondary lysosomes carrying various cytoplasmic organelles (Fig. 6A and B). These structures are found in large numbers in senescent cells in vivo as well as in aging cells of diverse organisms and are recognized as morphological signs of aging (41, 42).

Antioxidant Status Is Reduced in TGFa/c-myc Livers—GSH plays a central role in cellular defense against oxidative stress (43). GSH regulates the intracellular concentration of ROS via reactions catalyzed by GPx (44). We thus determined whether modulation of GSH levels and activity of cytosolic GPx, the major glutathione peroxidase isozyme present in liver (45), contribute to development of oxidative stress in TGF-a/c-myc

Fig. 4. TGF-a/c-myc hepatocytes exhibit signs of fatty liver phenotype. A, ultrastructure of centrolobular hepatocyte from 10-week-old WT mouse; B, double transgenic hepatocyte in the vicinity to central vein (CV) shows increased lipid deposition (L). Original magnification, × 4000.
livers. In WT mice, the amount of cellular GSH was about 35% lower (p < 0.001) in 5-week-old than in 10-week-old livers (Fig. 7). These results indicate that the GSH system is not fully developed in the maturing mouse livers at 5 weeks. Both transgenic lines showed a similar pattern of expression of cellular GSH during liver ontogenesis, but the amount of GSH was consistently lower in the transgenic animals as compared with WT controls (Fig. 7). In contrast, the activity of GPx, a key antioxidant enzyme, was decreased only in TGF-α/c-myc and not in c-myc livers (Fig. 8A), concomitant with elevation in lipid peroxidation (Fig. 2). It is noteworthy that there was no significant difference in the activity of GPx in liver samples from WT and c-myc mice of different ages. However, the activity of GPx increased in TGF-α/c-myc livers in an age-dependent manner. This induction of GPx activity may be an adaptive response to compensate for increased oxidative stress in double transgenic livers consistent with reports in aging livers (46, 47). Interestingly, the tumors which developed in TGF-α/c-myc livers displayed very little GPx activity (16% of peritumoral levels) (Fig. 8A). We next examined the activity of thioredoxin reductase, a cellular selenocysteine-containing protein (48) which together with thioredoxin and NADPH functions as a powerful protein disulfide-reducing system (9, 49, 50). In contrast to GPx, thioredoxin reductase activities in WT, c-myc, and TGF-α/c-myc mice were essentially the same. However, hepatic tumors in TGF-α/c-myc mice exhibited a marked induction (about 30%) of thioredoxin reductase activity (Fig. 8B).

**DISCUSSION**

In the present study we have demonstrated that TGF-α/c-myc hepatocytes exhibit a striking increase in intracellular peroxide production as estimated by the peroxide-activated fluorescent dye DCFH-DA (32, 37, 38). The elevation in ROS production was not an immediate outcome of TGF-α and c-myc transgene expression. The effect was not evident at 5 weeks of age but was typically observed by 10 weeks when the generation of ROS was enhanced by 3- and 8-fold in c-myc and TGF-α/c-myc mice, respectively. This delayed effect might reflect a persistent level of oxidative damage which overwhelms constitutive cellular antioxidant defense mechanisms and eventually results in oxidative stress and liver damage. Peroxidation of unsaturated fatty acids in membrane phospholipids is one of the multiple cytotoxic effects of oxidative stress (39). Consistent with this, lipid peroxidation was approximately 2-fold higher in TGF-α/c-myc livers than in either WT or c-myc livers.

**FIG. 5.** PCR detection of deletion in liver mtDNA. Total hepatic DNAs from WT, c-myc and TGF-α/c-myc mice at 5 and 10 weeks of age were subjected to PCR amplification and separated using a 1% Tris-acetate-EDTA-agarose gel electrophoresis as described under “Experimental Procedures.” The position of PCR deletion product of 851 bp is indicated.

**FIG. 6.** Ultrastructural manifestation of premature aging in TGF-α/c-myc transgenic hepatocytes from 10-week-old mouse. Tissue was processed for transmission electron microscopy as described under “Experimental Procedures.” Lipofuscin granules are indicated by asterisks (A and B), and the secondary lysosome (open arrow)-containing remnant of mitochondria and fragment of lipid (B) can be clearly seen within the cell. Mitochondrion is indicated by a thin arrow. Original magnification, × 20,000.

**FIG. 7.** Concentrations of total glutathione in the livers of WT, c-myc, and TGF-α/c-myc mice of different age. Data are expressed as mean ± S.E., n = 5 mice in each group. *p < 0.01 when compared with age-matched WT or c-myc mice; **p < 0.001 when compared with corresponding mice at 5 weeks of age by Student’s t test.
Loss of GPx activity has been shown to be due to depression of enzyme biosynthesis and occurred in parallel with suppression of phospholipid-hydroperoxide glutathione peroxidase4 which is involved in the degradation of products of the multiple lipoxygenases (44). The latter observation broadens the family of possible oxidants to include a large variety of organic hydroperoxides, and points to the critical importance of hydroperoxide turnover in the development of oxidative stress in TGF-α/c-myc livers. In addition, constitutive expression of GSH itself was slightly reduced in TGF-α/c-myc mice as well as in the c-myc mice. The comparable decreases in GSH levels in both transgenic models indicate that the rate of ROS generation is more critical than the lowering of GSH levels as a determinant of the overall oxidized state in the TGF-α/c-myc hepatocytes. However, the finding of a relatively small decrease in total hepatic GSH content might obscure a substantial localized depletion in a sub-population of cells. This possibility is supported by numerous observations related to the TGF-α/c-myc model. First, the livers of TGF-α/c-myc mice demonstrated a dysplastic phenotype in the form of nuclear atypia and remarkable hypertrophy initially evident in the pericentral regions of hepatic parenchyma where concentrations of total reduced GSH are usually smaller (53). Second, up-regulation of TGF-β1 and urokinase-type plasminogen activator gene expression was detected in the centrolobular areas of hepatic parenchyma (27) concurrent with increased ROS production in TGF-α/c-myc livers, although we do not know if these phenomena are interdependent. There is evidence that oxidation might be a mechanism of inactivation of certain protease inhibitors including plasminogen activator inhibitor (54, 55) as well as activation of latent TGF-β1 (56). On the other hand, the ability of TGF-β1 to stimulate cellular production of H₂O₂ in a variety of cell types (18–22), including hepatocytes (23, 24, 57), has been well established. Moreover, TGF-β1 has been reported to suppress the expression of certain antioxidant enzymes such as catalase and superoxide dismutases in rat hepatocytes (58). Taken together, these findings suggest an involvement of ROS in the development of dysplasia, the hallmark of early pathological changes in TGF-α/c-myc livers. Of potential importance is the observation that 4-HNE, a representative hydroxylalkenal formed during lipid peroxidation with a wide spectrum of biological effects (39, 51, 59, 60), has been found to induce the expression of both mRNA and TGF-β1 protein in cells of macrophage lineage (61).

A close correlation exists between the degree of ROS overproduction and occurrence of liver tumors in transgenic mice. The increase in the cellular oxidative stress was more pronounced and sustained in TGF-α/c-myc than c-myc mice, consistent with our carcinogenic data (26, 27). Although constitutive expression of c-myc alone was sufficient to increase intracellular ROS albeit to a lesser extent, c-myc expression did not result in increase in lipid peroxidation or specific chromosomal damage at 10 weeks (28). However, in the c-myc as well as TGF-α/c-myc livers, there were significantly increased amounts of mtDNA deletion starting from 5 weeks of age. MtDNA is known to be 10–15-fold more sensitive to oxidative damage than is nuclear DNA (40, 62). Enhanced ROS generation and the progressive accumulation of mtDNA damage have been increasingly described in aging rodent and human livers as well in degenerating diseases associated with oxidative liver damage (33, 63–69). Further characterization of double transgenic hepatocytes showed the presence of lipofuscin granules, a pigment ascribed to the aging process and thought to derive from lipid peroxidation (7). These observations suggest that increased ROS generation in c-myc and TGF-α/c-myc hepato-
cytes may lead to premature oxidative damage of hepatic mtDNA. Although it remains to be determined, it seems possible that overexpression of TGF-α transgene alone might also promote ROS production. However, a considerably lower level of chromosomal damage (28) and a longer latency of liver tumor development is observed in TGF-α (31) and in c-myc single transgenic mice (27). This suggests that co-expression of TGF-α and c-myc transgenes might synergistically augment ROS production and/or decrease the antioxidant defense, resulting in the acceleration of carcinogenesis in TGF-α/c-myc double transgenic mice.

Importantly, TGF-α/c-myc hepatocytes displayed both an increased oxidant generation and a higher rate of cell proliferation than c-myc single transgenic mice (30, 70). The coupling of these two phenomena increases the risk of mutagenesis as well as fixation and propagation of the mutation (71), and might be responsible for the excessive chromosomal damage and acceleration of hepatocarcinogenesis in TGF-α/c-myc transgenic mice. Indeed, by 2 months of age, TGF-α/c-myc hepatocytes displayed a wide spectrum of chromosomal abnormalities, including chromosomal breaks, translocations, endoreduplication, and aneuploidy (28), similar to effects of ionizing radiation. Moreover, enlarged dysplastic TGF-α/c-myc hepatocytes accumulated more severe DNA damage concurrent with increases in DNA content and loss of replicative potential (28–30). A similar senescence-like phenotype characterized by growth cessation and marked cellular enlargement has also been observed in cultured fibroblasts treated with H2O2 (72–74). Furthermore, H2O2 was capable of inducing complete growth arrest and/or apoptosis depending on concentration (74). Interestingly, dysplastic hepatocytes in TGF-α/c-myc livers increased in number after a period of rapid cell proliferation, underwent apoptosis with a high frequency (27, 70) and the acceleration of carcinogenesis in TGF-α/c-myc hepatocytes may lead to premature oxidative damage of hepatic mtDNA.

Our knowledge these studies provide the first biochemical evidence that co-expression of TGF-α and c-myc transgenes in mouse livers results in overproduction of ROS. These data support the hypothesis that chronic stimulation of cell proliferation in liver facilitates the creation of an environment of oxidative stress leading to massive DNA damage and acceleration of hepatocarcinogenesis in this animal model. Further studies are necessary to elucidate the signaling pathways that regulate ROS generation and the nature of the specific ROS responsible for genetic damage.

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