its hepatocarcinogenicity in rodents the dose dependence of its promoting effects was first investigated by Masuda *et al.*[15] in a medium-term rat liver bioassay (Ito test). When F344 male rats were given α-BHC at a wide range of doses from 0.01 to 500 ppm in the diet for 6 weeks after a single intraperitoneal injection of DEN, quantitative values for numbers and areas of GST-P positive foci were dose-dependently increased at 0.5 to 500 ppm. However, a tendency for a decrease was observed with 0.01 and 0.1 ppm α-BHC (Fig. 3). As observed with phenobarbital, CYP3A2 protein levels and activities showed a good correlation with the numbers and areas of GST-P positive foci. This experiment provided supportive evidence for hormesis in the promotion by α-BHC of rat hepatocarcinogenesis and suggested that the mechanism might be related to the suppression of P-450 isoenzyme CYP3A2 protein expression by low doses[15].

A second study was conducted with α-BHC applied to F344 rats at doses of 0.01 to 500 ppm for 10 weeks after DEN initiation[57]. While α-BHC promoted the formation of GST-P positive foci at the dose of 500 ppm, both the numbers and areas of preneoplastic lesions were found to be significantly reduced with 0.05 ppm. The dose response curves for cytochrome P-450 content, NADPH-cytochrome P-450 reductase activity and 8-OHdG formation exhibited essentially the same patterns as for GST-P positive foci. A low dose of α-BHC also tended to up-regulate Ogg1 mRNA expression. Similar to the phenobarbital case, α-BHC treatment lead to increase in PCNA positive cells within the areas of GST-P positive foci at a dose of 500 ppm but decreased values at low doses. Though the response curves for CYP2B1 and 3A2 catalytic activity, protein levels and mRNA expression showed thresholds, CYP2C11 activity exhibited an inverted J-shape. This major constitutive male-specific isoform was thus found to be up-regulated by a low dose of α-BHC treatment at the transcriptional level and with regard to catalytic activity detected with 2α- and 16α-testosterone metabolites. Thus, CYP2C11 might take part in detoxification while CYP2B1 and 3A2 isoenzymes are considered to participate in bioactivation of α-BHC and increase its toxicity, given the correlation with GST-P positive foci and oxidative DNA damage. The non-linear threshold dose response observed at low doses with respect of CYP2B1 and 3A2 can be deemed a result of a multi-step process "turning on" orphan nuclear receptors, constitutive androstane receptors and the pregnane X receptor, which is known to regulate CYP2B1 and 3A2 transcription by binding as a heterodimer to the retinoid X receptor[58,59]. Furthermore, in the same study it was shown that glutathione-S transferase, which plays an important role in detoxifying α-BHC, demonstrates a threshold in its activity towards α-BHC at low doses[57,60].

The possibility of a hormetic effect of α-BHC regarding formation of liver tumors *in vivo*, was further examined in F344 rats at doses from 0.01 to 500 ppm in the diet for 36 weeks after initiation of hepatocarcinogenesis with DEN (unpublished data). Incidences and multiplicities of liver

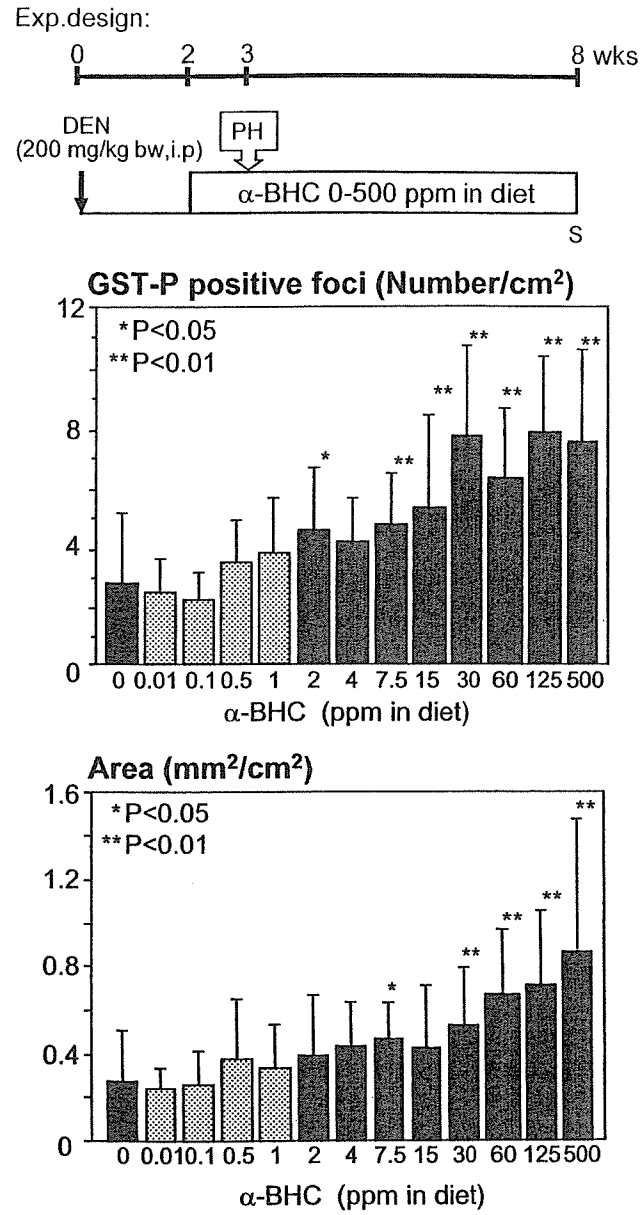

Fig. 3. Induction of GST-P positive foci in the liver of rats treated with α-BHC in a medium-term bioassay. PH, 2/3 partial hepatectomy. S, sacrifice.

tumors were found increased in a dose-dependent manner by α-BHC at doses of 0.5–500 ppm, while a tendency for decrease in their values was found in the low dose 0.01 and 0.1 ppm groups, similar to the case with rat liver preneoplastic lesions.

From these results it was concluded that α-BHC indeed exhibits hormesis regarding its hepatocarcinogenicity by mechanisms involving induction of detoxifying enzymes at low dose, as well as influencing free radical production and oxidative stress, and consequently pathological change in the liver. In these studies, the dose response relationship for GST-P positive foci was represented by a J-shape curve, in line with the previous investigation of this chemical using

the Ito test[15].

Possibility of a Hormesis for Hepatocarcinogenicity of DDT

Inhibitory effects on the induction of GST-P positive foci were also noted with low doses of another non-genotoxic carcinogen, DDT[28]. First, in the study of Sukata *et al.*, F344 rats, 21-day-old at the commencement, received DDT at doses from 0.005 to 500 ppm in the diet for 16 weeks. In another experiment Kushida *et al.*[61] investigated the possibility of hormesis after DDT administration to F344 rats for 11 and 43 weeks following initiation of hepatocarcinogenesis with DEN. In both experiments the doses of 20 ppm and above were associated with dose-dependent induction of GST-P positive foci in the liver. In contrast, 0.005 and 0.01 ppm administration resulted in a tendency for decrease in values below the control level (Fig. 4). Histopathological analysis of liver nodules also revealed a tendency for decrease in the incidence and multiplicity of hepatocellular carcinomas in the low dose groups as compared to the DEN initiation controls. The multiplicity of total tumors also tended to decrease, although incidences were similar. Alteration of the GST-P positive foci in the low dose groups was correlated with a tendency for decrease in the CYP3A2 protein level as well as induction of IL-1 receptor type I (IL-IRI) and TNF-α receptor type I, whose ligands have roles in downregulating CYP3A2 and influencing cellular proliferation or apoptosis[16]. IL-1R1 is known to be a cell surface molecule involved in cell signaling[62], while IL-1 inhibits regeneration of rat liver cells[63] and tumor cell growth[64], and inhibitory actions of IL-1β on hepatocyte DNA synthesis are effected by iNOS gene expression and NO production under IL-1R1 control[65].

It was found that within GST-P positive areas, cell proliferation was slightly lower in the 0.005 ppm DDT dose group than in the DEN only treated group[16]. As observed in experiments with phenobarbital and α-BHC, CYP2B1/2 and CYP3A2 protein levels in the liver microsomal fraction were significantly elevated by high doses of DDT. In line with previous results, 8-OHdG formation was significantly suppressed by a low dose of the chemical, presumably related to effective DNA repair and co-repair of endogenous damage, which may exceed formation of adducts[61]. Oxidative stress in the low dose group was suggested to be decreased because of the lowered CYP3A2 expression and formation of 8-OHdG balanced through elimination by Ogg1[16,61]. Furthermore, in the low DDT dose group, mRNA expression and immunohistochemical staining of connexin 32 (Cx32) were found to be elevated[16]. Many previous studies indicated that high doses of DDT and other non-genotoxic carcinogens inhibit Cx32, resulting in the loss of the function of gap junction intracellular communication (GJIC) and release of potentially initiated cells from growth constraints imposed by normal neighboring cells, resulting in clonal expansion and ultimately tumor formation and progression[66–70]. In the present study, mRNA expression of

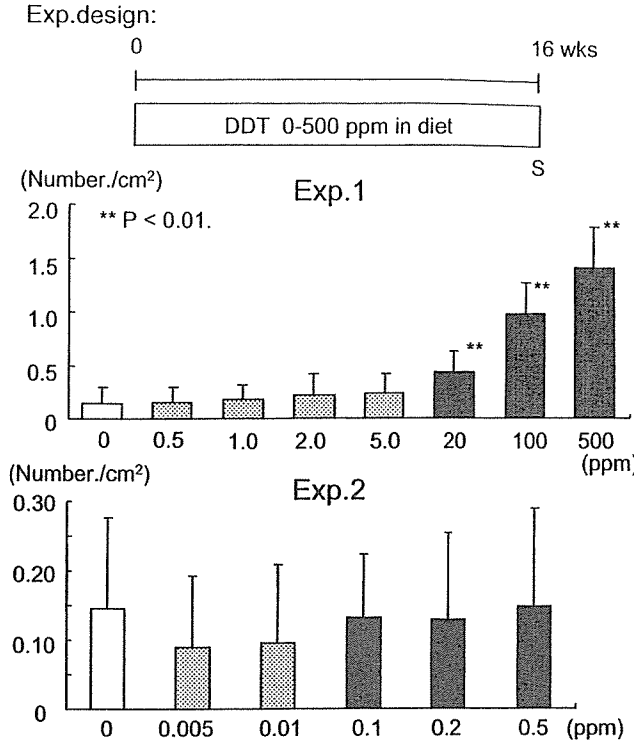

Fig. 4. Induction of GST-P positive foci in the livers of rats treated with DDT for 16 weeks. S, sacrifice.

one of the transcriptional factors, HNF-1α, which regulates Cx32 expression[71,72], was in good correlation with that of Cx32[61]. Differential alteration of HNF-1α is suggested to be one of the possible mechanisms by which DDT might inhibit or promote rat hepatocarcinogenesis.

Hormetic Effects Observed with Ethanol

Effects of alcohol intake on cardiovascular diseases[73], stroke[74], all causes of death[73,75], and cancer mortality[76] are known to demonstrate U- or J-shaped curves; that is, those who consume a little alcohol have the lowest risk. The relationship between smoking or drinking dose and risk for stomach cancer has also attracted great interest as to whether strict dose-dependence or a U-shaped curve might be evident[77]. Recently, the risk of stomach cancer was reported to increase linearly with the smoking dose, but not with the drinking dose. Kikuchi *et al.*[78] showed that light drinkers in Japan have the lowest risk of developing stomach cancer among both male and female subjects, and heavy drinkers the highest risk among males, the association being J-shaped among male subjects, and U-shaped among female subjects, and thus very similar to the association with risk of cardiovascular diseases and stroke. J- or U-shaped dose-responses were suggested to offer an explanation for the fact that more studies on stomach cancer have demonstrated an association with smoking than with drinking[78].

In a recent study the promoting effects of ethanol at different doses on MeIQx induced liver carcinogenesis in

F344 rats was evaluated[79]. No significant inhibitory activity on hepatocarcinogenesis was observed after administration of ethanol at low doses (0.1–1%), while a high dose of ethanol (10–20% in drinking water) was found to exert clear promotion of development of MeIQx induced liver cancer in rats.

Adaptive Mechanisms

To explain hormetic effects, adaptive responses have been proposed. When experimental animals are exposed to biologically effective levels of chemicals, their bodies have to deal with chemical perturbation and diverse responses are elicited. For some chemicals, the initial response constitutes an adaptive effect that maintains homeostasis[19,21]. Disruption of this balance at any level of organization may lead to an adverse effect, or toxicity. When target cells are exposed to non-genotoxic carcinogens, as described above, it is to be expected that machinery to conserve homeostasis would be switched on, for detoxification and excretion, with preservation of the cell cycle and programmed cell death regulation through cell signaling. At very low doses of chemicals, such mechanisms in target cells might more than compensate for cell injury, so that not only a dose threshold but also a reduction in lesion development, as compared to the control case, may occur. This would explain the U- or J-shaped response curves obtained for phenobarbital, α-BHC and DDT hepatocarcinogenicity (Fig. 5).

Hepatic adaptive responses usually involve actions of the chemical on cellular signaling pathways, often receptor mediated, leading to changes in gene expression and ultimately alteration of the "metabolome", directed toward maintaining homeostasis through modulation of various cellular and extracellular functions. At all levels of organization, adaptive responses are beneficial in that they enhance the capacity of all units to respond to chemical induced stress, are reversible and preserve viability. In contrast, adverse or toxic effects produced by genotoxic chemicals often involve chemical reactions with cellular macromolecules such as DNA or proteins and result in disruption of homeostasis. Such effects can be nonreversible at all levels of organization resulting in mutations or inactive protein molecules. Examples of compounds eliciting adaptive effects are provided by phenobarbital and ciprofibrate, whereas p-dichlorobenzene and 2-AAF, for instance, exhibit primarily toxic effects.

Hormetic Effects with Endogenous ROS

Exposure to different chemical carcinogens for which hormetic effects are proposed leads to formation of ROS, and frequently to induction of cytochrome P-450 species, with induction of oxidative stress. ROS are genotoxic in principle, and the question arises as to whether chemicals that increase ROS production will add to an endogenously produced background level of DNA lesions, or whether compensatory mechanisms exist that may result in non-

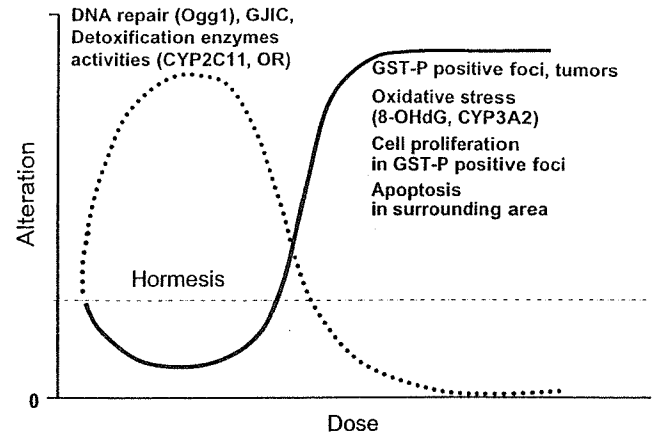

Fig. 5. Potential mechanisms mediating hormesis in carcinogenesis.

linear dose-effects. Endogenous ROS cause detectable background levels of DNA damage, namely in the form of oxidized bases (e.g. 8-OHdG), apurinic (AP) sites, and strand breaks. Oxygen radicals also attack other cellular components such as lipids, to generate reactive intermediates that couple to DNA and give rise to exocyclic etheno- and propane-adducts and 1,N^6-ethenodeoxyguanosine and 3,N^4-ethenodeoxycytidine[80–82]. Such adducts will have mutation-associated consequences upon cell replication[83]. The continuous production of free radicals from radiation and other sources has stimulated organisms to evolve repair systems for oxidative base modifications or chromosome breaks. Alteration to DNA molecules triggers repair, and frequent activation may increase the general repair capacity, irrespective of the cause of the damage. Repeated exposure to ROS may thus lead to an adaptive response, mitigating the mutagenicity of oxidative DNA lesions. DNA repair is a crucial factor in maintaining a low steady-state level of DNA damage and its impairment is implicated in processes that promote human cancer[84]. It is difficult to state at the present time the precise role of ROS-induced DNA damage in carcinogenesis and how genetic and epigenetic events induced by ROS interact with cell transformation and malignant progression. However, many aspects have already been elucidated, indicating that at low levels of ROS, adaptive responses, repair and antioxidative defenses are strengthened, whereas at high levels they may be overwhelmed. Whether induction of a detoxifying enzyme qualifies as a basis for a practical threshold depends on the speed and capacity of removal of the reactive species from the system compared with the speed of the translocation of the reactive species from the site of its generation to the nucleus and reaction with the DNA.

Bystander Effects

Numerous investigations have revealed that several cancer relevant effects of ionizing radiation can occur in cells that have received only cytoplasmic or plasmalemmal

membrane exposure to ionizing radiation[85-92]. Furthermore, many effects that have been attributed to ionizing radiation-induced damage to nuclear DNA or that occur following irradiation of the cytoplasmic compartment of cells can also occur in cells that have received no direct exposure to ionizing radiation. These so-called "bystander effects" as well as adaptive responses are linked to biological effects of radiation and chemical treatments and involve intracellular communication systems (both gap junctional and extracellular communication)[85]. Bystander effects are considered to be induced by radiation in non-irradiated cells when an extracellular signal produced by a radiation-targeted cell is received by a non-hit cell, or by gap junctional direct transfer of some radiation-induced signals[86]. Bystander effects may include increase in intracellular ROS, induction of mutations, enhanced cell growth, apoptosis, genomic instability and neoplastic transformation, as well as cell death[87-92]. Both direct transfer of small molecules or ions through gap junctions and extracellular signaling by secreted factors (hormones, cytokines, growth regulators, etc.) maintain homeostasis and might be related to hormesis[86]. The implications of bystander effects of low and high dose radiation exposure for potential health endpoints still need to be resolved.

Dose Response in Cell Proliferation, Apoptosis and DNA Repair

Induction of ROS has been observed to alter cell proliferation and apoptosis in the tissues. While marked increase in oxygen radicals in the rat liver in cases of non-genotoxic carcinogens phenobarbital, α-BHC and DDT at high dose, for example, leads to elevation of PCNA indices in areas of GST-P positive foci, cell proliferation rates at low doses were found to be decreased[14]. Suppression of liver nuclear DNA 8-OHdG formation at low dose may be associated with reduction of cell proliferation within GST-P positive foci. Furthermore, apoptosis, significantly induced by high dose administration in liver tissue surrounding GST-P positive foci, was suppressed in the low groups, with strong similarity to the pattern observed for 8-OHdG. Apoptosis of normal-appearing liver tissue has been proposed as one factor regulating the size of foci, as enlargement of GST-P positive foci presumably requires regenerative stimuli. In a low dose phenobarbital study, the results of cDNA microarray analysis indicated 2 ppm to specifically enhance mRNA expression for glutamic acid decarboxylase (GAD65), an enzyme involved in the synthesis of gamma-aminobutyric acid (GABA), while suppressing expression of MAP kinase p38, JNK1, 2 and other intracellular kinases[14]. A negative correlation between the expression of GABA-A receptors in hepatocytes and thymidine incorporation in liver specimens was reported, albeit without evidence of a causal relationship, and the GABA-B receptor subtype is known to be involved in hepatocyte DNA synthesis and mediation of growth stimulation[93,94]. Thus, the suppression of gene expression of

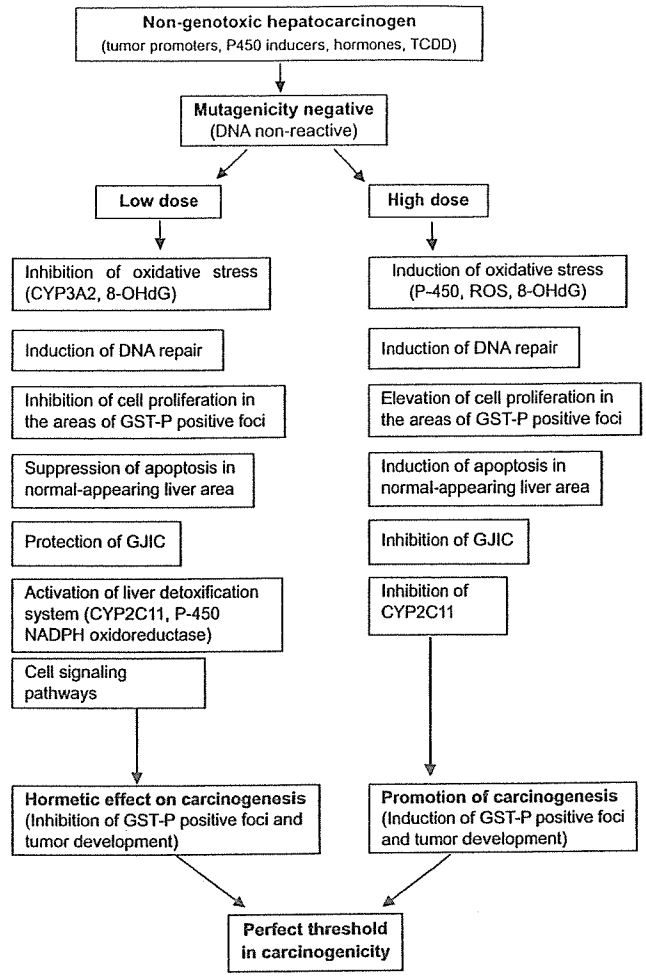

Fig. 6. Proposal of a flow scheme toward dose-effect relations, risk assessment and mechanisms of action of non-genotoxic chemical carcinogens.

signal transduction modulators, such as MAP kinase p38, JNK1, 2 and other intracellular kinases might be a factor related to the inhibitory effect of phenobarbital on cell proliferation.

The fact that DNA repair protects cells from fixation of DNA damage in the newly synthesized DNA strand as heritable mutations, means that outcome of exposure to carcinogens is dependent on the race between repair and proliferation-dependent DNA synthesis. The combination of elevated repair and decreased cell division may more than compensate for deleterious influence. Application of higher doses of the same substance may result in an increased tumor incidence because of cell cycle progression due to cytotoxicity and regenerative cell proliferation. As a consequence, a J-shaped dose-effect curve results. It is proposed that cell cycle progression and regenerative proliferation represent the key parameters concerning threshold mechanisms, although apoptosis also contributes. This would be particularly important for epigenetic carcinogens, whereas the genotoxic substance levels of DNA

damage in target tissues are far higher. Furthermore, it should be borne in mind that apoptosis and the control of neoplastically transformed cells by the immune system may be additional factors influencing the shape of the dose-effect curve.

Conclusions

In summary, recent data on the effects of non-genotoxic carcinogens, indicate the existence of hormesis and a "perfect" threshold for carcinogenicity (Fig. 6). Hormesis by non-genotoxic carcinogens implies the maintenance of homeostasis, with adaptive responses involving cell proliferation and apoptosis, DNA damage and repair, cell signaling, and cell-cell communication. The findings have broad implications for cancer risk assessment methods, experimental design, and the establishment of optimal drug doses, taking advantage of adaptive effects. Quantitative analyses based on biological models are necessary, with attention to factors that affect the degree of non-monotonicity. Further analyses along these lines should promote scientific discussion of biphasic dose responses and the concepts of "hormesis" and thresholds, particularly for tumor induction by non-genotoxic carcinogens.

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Aldo-keto reductase 1 family B7 is the gene induced in response to oxidative stress in the livers of Long-Evans Cinnamon rats

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Abstract. The Long-Evans Cinnamon (LEC) rat strain (*Atp7b* m/m), which accumulates copper in the liver due to mutations in the *Atp7b* gene, is a useful model for investigating the relationship between oxidative stress and hepatocarcinogenesis. To determine the effect of this mutation on oxidative stress marker genes, we performed oligonucleotide array analysis (Affymetrix), and compared the results in *Atp7b* m/m rats with those of a sibling line with the *Atp7b* w/w genotype. We focused our studies on the expression of the aldo-keto reductase 1 family B7 (AKR1B7)-like protein gene, since this gene codes for reductase enzymes involved in the detoxification of oxidizing compounds (e.g., aldehydes) and was differentially expressed in *Atp7b* m/m and *Atp7b* w/w rat liver. Akr1B7 mRNA expression was significantly increased in comparison with the expression of 4 other known oxidative stress responsive genes, haem-oxygenase-1 (HO-1), thioredoxin (Trx), aldehyde reductase (AKR1A1), and glucose-6-phosphate dehydrogenase (G6PDH). By searching binding motifs, five nuclear factor kappa B (NF-κB) binding sites were located in the 5'-upstream region of the *Akr1b7* gene. Transient co-transfection with both *I-κBα* and the Akr1b7 6 kb promoter (p6.0-AKR-Luc) inhibited luciferase activity of p6.0-AKR-Luc in HepG2 cells. Cuprous ion however did not affect the transcription activity induced by p6.0-AKR-Luc. Gel-shift assay showed that the DNA binding activity of NF-κB increased in the livers of LEC rats, suggesting that the oxidative stress is mediated through NF-κB. The results indicate conclusively that in LEC rat liver, *Akr1b7* might be up-regulated by oxidative stress mediated through NF-κB, but not that mediated directly by copper.

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Abbreviations: AKR1A1, aldo-keto reductase family 1, member A1; AKR1B7, aldo-keto reductase 1 family B7; ATP7b, ATPase7b; EpRE, electrophile response element; EST, expressed sequence tag; G6PDH, glucose-6-phosphate dehydrogenase; GST-P, glutathione S-transferase-placental; HO-1, haemoxygenase-1; 8-OHdG, 8-hydroxydeoxyguanosin; IκBα, inhibitory kappa B alpha; LEC, Long-Evans Cinnamon; MT, metallothionein; NF-κB, nuclear factor kappa B; PUFA, polyunsaturated fatty acid; Trx, thioredoxin

Key words: AKR1B7, oxidative stress, oligonucleotide array, real-time PCR, Long Evans Cinnamon rat

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and one of the most deadly cancers with approximately 600,000 yearly deaths (1). Hepatocarcinogenesis is a multi-step process and cellular oxidative stress is one of the factors responsible for the propagation of liver diseases, such as hepatitis, cirrhosis, and hepatoma (2-4). Although much is known about the cellular pathogenesis and etiological agents leading to HCC, the molecular events are not well understood. Recent advances in genomics and proteomics have had the potential to identify characteristics of HCC that may be related to prognosis or to etiology, and these may be useful in HCC screening or diagnosis. Here the Long-Evans Cinnamon (LEC) rat model provides a unique opportunity to unveil the early molecular event associated with oxidative stress-related hepatocarcinogenesis.

The Long-Evans Cinnamon (LEC) rat strain, which accumulates copper in the liver due to mutations in the *Atp7b* gene, has been used as a model to investigate the relationship between oxidative stress and hepatocarcinogenesis (5-11). The hepatic levels of oxidative stress indicators in LEC rats were found to be elevated during the onset of fulminant hepatitis between 16 and 24 weeks of age, while the levels of antioxidants such as ascorbate and ubiquinol were concomitantly decreased. Oxidative stress levels in LEC rats were highest at the onset of hepatitis, which occurred when the rats were around 20 weeks of age (Fig. 1.) The oxidative stress levels

decreased from peak levels but still remained elevated during the subsequent phases of chronic hepatitis and hepatocellular carcinoma in LEC rats. High oxidative stress levels in the liver of LEC rats at the hepatitis stage may be involved in the subsequent development of liver cancer. Therefore, the LEC rat model can be considered to be useful for exploring oxidative stress-responsive genes associated with the development of neoplastic lesions.

Thus, the present study examined the gene expression patterns associated with oxidative stress by utilizing an oligonucleotide array containing more than 8732 expressed sequence tag (EST) clusters. The difference in gene expression between *Atp7b* m/m and *Atp7b* w/w rats was analyzed. Based on the gene array results, candidate genes were further evaluated by RT-PCR and real-time PCR. We found that the gene whose expression was most altered in response to oxidative stress was *Akr1B7*, and we describe the expression and transcriptional regulation of this gene in the present study.

Materials and methods

Chemicals and animals. Taqman reverse transcription reagents and Taqman 1000 Rxn Gold/Buffer A were provided by Applied Biosystems Roche (Branchburg, New Jersey, USA). RNeasy mini kit was from Qiagen (Tokyo, Japan). GeneChip rat genome RG-U34B set was obtained from Affymetrix (Santa Clara, CA, USA). *Atp7b* w/w (wild/wild) and *Atp7b* m/m (mutant/mutant) in siblings of LEC x (F344xLEC)F1 backcrossed rats (20 weeks old) were used for oligonucleotide array analysis to minimize background noise due to factors such as strain differences. *Atp7b* m/m rats have the same biological features as LEC rats with respect to hepatic copper accumulation and the onset of fulminant hepatitis (10). For quantitative determination of gene expression, to verify the array data, 24- to 48-week-old male LEC rats with the *Atp7b* m/m genotype and healthy LEA rats with the *Atp7b* w/w genotype were used. The livers were dissected, snap-frozen in liquid nitrogen, and stored at -80°C until use.

Oligonucleotide array and RT-PCR. RNA from 3 rats with either the *Atp7b* w/w or *Atp7b* m/m genotype (age, 20 weeks) was pooled. Total RNA was extracted from frozen livers using an RNeasy kit and DNase kit according to the manufacturer's protocols (Qiagen, Chatsworth, CA). The GeneChip rat genome RG-U34B set (Affymetrix, Santa Clara, CA) was used according to the protocol provided. Data were analyzed using GeneChip expression analysis software. Expression changes were reflected in the differences in signal intensity from array hybridization. A two-fold or greater change in signal intensity was considered a significant change in expression.

For RT-PCR, Taqman reverse transcription reagents and Taqman 1000 Rxn Gold/Buffer A were used for cDNA synthesis and PCR, respectively according to the manufacturer's protocols. All signals were normalized against the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene, which was amplified from the same dilution series. Primers used for RT-PCR were as follows: *Akr1b7*, 5'-TGAGAGTGA GGTGGGAGAA-3' and 5'-TCCTCGTGGAAAGGAAAGT CC-3'; glucose-6-phosphate dehydrogenase (*G6PDH*), 5'-AT

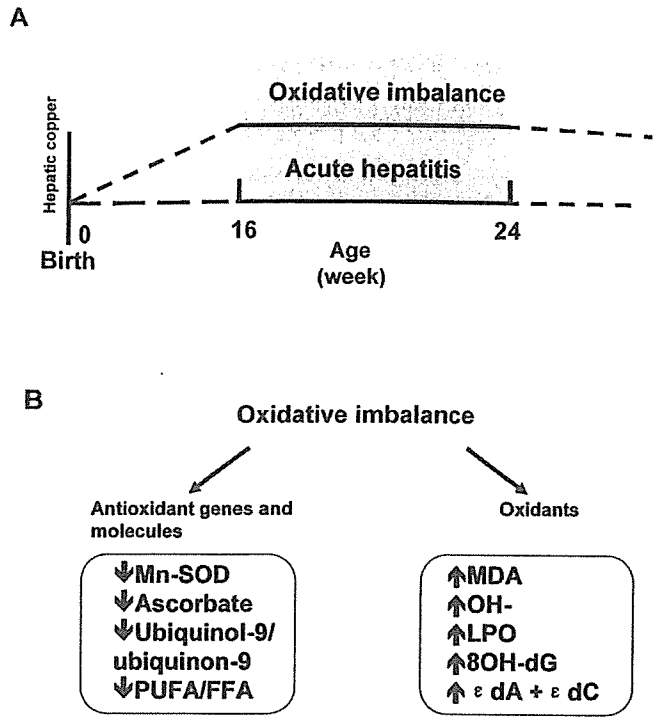

Figure 1. A, a high level of oxidative status persisted in livers of 16- to 24-week-old LEC rats due to copper accumulation. B, oxidative imbalance may cause a decrease in antioxidant biomarkers and an increase in oxidant levels. Studies on these biomarkers have been published before by our group and by others. Hydroxyl free radical (OH•) (5), LPO (6), etheno adducts (sum of dA + dC) (8), 8-hydroxy-deoxyguanosin (8-OHdG) (7), and malondialdehyde (MDA) (8) are products of oxidation. Ascorbate, superoxide dismutase, polyunsaturated fatty acid: free acid ratio (PUFA/FFA), and ubiquinol-9 (9) are antioxidants. In LEC rats at the age of 16 to 24 weeks old, the levels of oxidative products including MDA, OH•, 8-OHdG and εdA + εdB, were significantly higher than those of age-matched healthy LEA rats (controls); however, the reductant ability, including that of Mn-SOD, ascorbate, ubiquinol-9/ubiquinon-9 and PUFA/FFA was decreased significantly when compared with LEA rats, which indicated that the redox status was unbalanced and that oxidative stress levels peaked in LEC rats during this period.

GGCCTTCTACCCGAAGACA-3' and 5'-TCATGTGGCTG TTGAGGTGCT-3'; haem-oxygenase 1 (*HO-1*), 5'-GTGTCC AGGGAAGGCTTTAAGC-3' and 5'-TGCTGTGTGGCTGG TGTGTAAG-3'; Thioredoxin (*Trx*), 5'-GCCAAAATGGTG AAGCTGATC-3' and 5'-AACATCCTGGCAGTCATCCA C-3'; aldehyde reductase (*Akr1a1*), 5'-AGATGCCTCTGAT TGGTCTGG-3' and 5'-TGGAGGTCACAAACAGCTCCT-3'.

Taqman real-time PCR method (12-13)

cDNA synthesis. cDNA was synthesized from total RNA (1 μg) in a final volume of 40 μl using Taqman reverse transcription reagents according to the manufacturer's protocol.

Primers and probes. Primers and probes that recognized rat cDNA sequences for *Akr1b7* and *G3PDH* were designed using Primer Express software (PE Applied Biosystems, Foster City, CA, USA). *G3PDH* was used as an endogenous control gene. The primers and probes used were as follows: *Akr1b7*, 5'-GA AGCAGATTTTAGCCACGTCTCT-3', 5'-GAGTCTGCAC CTTGATG-3' and probe 5'-CCTGGATGTAGGCTACCAT TTGGCCA-3'; G3PDH, 5'-GGCTGCCTTCTCTTGTGAC AA-3', 5'-CCGTGGGTAGAGTCATACTGG-3' and probe

5'-TGCCATCAACGACCCCTTCATTGAC-3'. All reactions were performed with an ABI Prism 7000 sequence detection system (PE Applied Biosystems). Each sample was amplified in triplicate. On the same plate, samples for standard curves for the target genes as well as a negative control (NTC) sample prepared without cDNA were included. The standard curves for the *Akr1b7* target gene and G3PDH were constructed with 10-fold serial dilutions of the target gene cDNA. The target messages in unknown samples were quantified according to the copy numbers by using the standard curves, and then normalized on the basis of G3PDH expression and expressed as copy number per 1000 copies of G3PDH.

Cloning of the 5' regulatory region of the rat Akr1b7 gene and construction of plasmids. The rat *Akr1b7* 6.0-kb 5' region (between nt -15 and -6000 from the transcription initiation site) was cloned by PCR with Advantage 2 polymerase mix (Clontech) in the presence of 1 μg of normal rat fibroblast NRF-49F genomic DNA as a template and 100 ng each of forward primer (5'-AGGAGACAGACTCACATAGCCCAA TG-3') and reverse primer (5'- GTTTGTGGACCTAAATAA CGCAGTG-3'). The PCR-amplified 6.0-kb DNA was first cloned into pGEM-T vector (Promega), and then digested with ApaI, followed by filling in with the large fragment of DNA polymerase I and SacI digestion. The resultant 6.0-kb *Akr1b7* DNA was cloned into SacI- and SmaI-digested pGL3 basic luciferase vector (Promega). The ARE-positive and -negative control reporters were derived from luciferase plasmids containing the 4.5 kb and 4.4 kb of the human ferritin H 5' region, respectively (14). ARE (antioxidant response element)-positive and -negative control reporters, hFHARE (+)-Luc and hFHARE(-)-Luc, were constructed from the 5'-upstream region of human ferritin H. Internal control reporter Renilla luciferase pRL-TK plasmid (the TK promoter region linked to the cDNA encoding Renilla luciferase) was purchased from Promega (Madison, WI), and I-κB expression plasmid pCMV-I-κBα was from BD Biosciences Clontech, Palo Alto, CA. All plasmids were purified by column chromatography (QIAfilter Maxi column, Qiagen, Santa Cruz, CA). The sequence of all plasmids was verified by automated sequencing.

Cell culture, transient transfection and luciferase assay. Human liver hepatocellular carcinoma HepG2 cells (ATCC# HB-8065) were maintained in Eagle's minimum essential medium supplemented with 100 units/ml penicillin, 10 μg/ml streptomycin, and 10% fetal bovine serum at 37°C under 5% CO_2. The transfections were carried out with Qiagen effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. To investigate the reporter activities in HepG2 cells, the total DNA transfected in each experiment was kept constant with reporter constructs (p6.0-AKR-Luc, Luc, hFHARE(+)-Luc, or hFHARE(-)-Luc; 100 ng per well), internal control Renilla reporter pRL-TK plasmid (2 ng per well), I-κB expression plasmid, or the empty vector pCMV to make the total amount of 152 ng. Prior to transfections, cells were plated in 24-well plates and grown overnight. The cells were transfected for 24 h, then washed, reincubated with fresh medium, and cultured for another 24 h. In the $CuSO_4$ and tBHQ treatment experiments, the cells were treated with or without $CuSO_4$ or tBHQ for 24 h at various concentrations. Cells were collected 24 h after transfection and the firefly and Renilla luciferase activities were measured with a dual-luciferase reporter assay system (Promega) on a Fluoroskan Ascent FL fluorometer (Labsystems, Franklin, MA). The firefly luciferase reporter activities were normalized by Renilla luciferase activities and expressed in relative light units (RLUs). The RLU of the p6.0-AKR-Luc or hFHARE(+)-Luc was set as 100%. The data were the mean ± SD from a minimum of 3 independent experiments with duplicates for each experiment and presented as RLU or percentage of activity.

Northern blot analysis. Total RNA (10 ug) was electrophoresed on a 1.5% denaturing agarose gel and then transferred to nylon membranes (Gibco BRL) and fixed by UV irradiation. The membrane was subsequently hybridized to an appropriate ^{32}P-labeled synthesized antisense oligonucleotide as probes (15). The Northern blot signals were visualized using a Storm 860 (Amersham Biosciences) scanner system.

Gel mobility shift assay. Preparation of nuclear extracts from liver was performed as described previously. Nuclear extracts containing 10 ug protein were incubated with 50 pg of 5'-end-labeled, double-stranded oligonucleotide (upper strand, 5'-AGTTGAGGGGACTTTCCCAGGC-3') for NF-κB, and subjected to a gel shift assay on a native 4% polyacrylamide gel, as described previously (16-17).

Immunohistochemistry for glutathione S-transferase placental form (GST-P)-positive cells. GST-P immunostaining was performed as previously reported (11). Briefly, sections were treated sequentially with rabbit anti-GST-P antibody (1;2000), biotin-labeled goat anti-rabbit IgG and the ABC staining system and then counterstained with methyl green. The numbers of GST-P-positive cells were counted manually over the entire portion of the cut surface of livers from 24-week-old LEC and LEA rats.

Statistical analysis. ANOVA with subsequent post hoc tests were used where appropriate. All values are expressed as the mean ± SD for 6 animals. Differences were considered significant at $p<0.05$.

Results

Oxidative stress in livers of LEC rats. Biochemical and pathological changes were measured to determine hepatic oxidative status in LEC rats. Fig. 2A shows Northern blots of the mRNAs for metallothionein (MT)-I, MT-II, and hsp73 mRNAs. The levels of both MT-I and MT-II mRNA in LEC rat liver were approximately 40-fold higher than those observed in age-matched LEA rat liver. Hsp73 (hsc70) is a member of the constitutively expressed heat shock proteins and is significantly induced by various stressors (e.g., heat-shock, heavy metals, oxidation). The hepatic levels of hsp73 mRNA in LEC rats were also significantly increased (~3-fold) as compared with LEA rats.

Fig. 2B shows the number of GST-P-positive cells detected by immunohistochemistry in the livers of LEC and LEA rats at 24 weeks of age. GST-P-positive (GST-P$^+$) cells were first observed at 20 weeks of age and reached a peak at 24 weeks

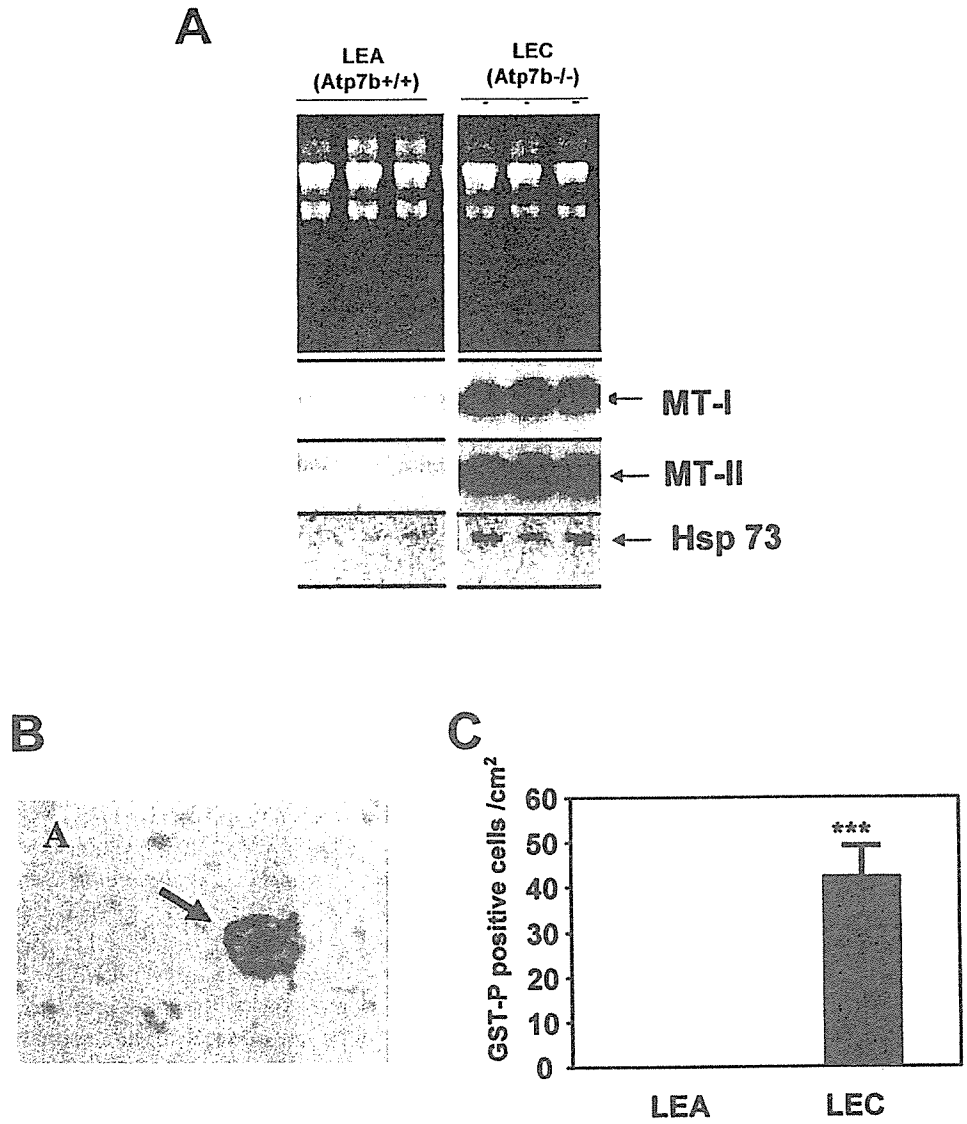

Figure 2. Biochemical and biophysical responses in LEC rat liver with the development of hepatitis. A, Northern blots of the mRNAs for metallothionein (MT)-I, -II, and hsp73 mRNAs. B, a typical picture of GST-P$^+$ cells in the liver. GST-P$^+$ cells were detected by immunohistochemistry using anti-GST-P antibody. C, the number of GST-P$^+$ cells per cm^2 in the livers of LEA and LEC rats aged 24 weeks of age. Data are expressed as mean ± SD of 7 rats. Student's t-test was performed for comparison of the number of GST-P$^+$ cells per cm^2. ***Significant difference at $p<0.001$ when compared with age-matched LEA rat group.

of age. No GST-P$^+$ cells were observed in livers of LEA rats at 24 weeks of age.

These observations regarding MT-I, MT-II, hsp73, and GST-P indicate that the livers of LEC rats at 24 weeks of age underwent high levels of oxidative stress.

Microarray analysis of LEC rat liver. In order to profile oxidative-responsive genes, RNA from the livers of LEC rats with hepatitis and age-matched wild-type rats was subjected to microarray analysis. Forty-five transcripts were up- or down-regulated in *Atp7b* m/m rat liver as compared with *ATP7b* w/w control rats. The differentially expressed genes were mainly related to oxidative stress, growth regulation, and transcriptional regulation. To verify the changes in gene expression observed in the array analysis, mRNA expression of these transcripts was further evaluated by RT-PCR. The relative expression detected by RT-PCR showed only ~38% concordance with the relative expression determined by gene array analysis. Nine transcripts showed higher mRNA expression in *Atp7b* m/m rat liver, whereas 8 transcripts showed lower expression as compared with *Atp7b* w/w rats (Table I). Known genes out of the 9 genes that were up-regulated were TRADD, Rat cofactor required for Sp1 transcriptional activation, subunit 6(CRSP6), 20s proteasome non-ATPase subunit mouse homologue, Tclone15/calcyclin, and human homologue MBD2 and MVDP/AKR1B7. Known genes out of the 8 down-regulated genes were CL-6, glucose-6-phosphatase catalytic subunit, and alternative transcript of mouse homologue growth hormone receptor. Out of the 17 up- and down-regulated genes, only *akr1b7* is associated with oxidation-reduction. Therefore, we next focused on transcriptional regulation of *Akr1b7* by oxidative stress in the livers of LEC rats.

Analysis of the correlation between akr1b7 and oxidative stress level. To further characterize the gene encoding AKR1B7 on

Table I. List of genes confirmed by semi-quantitative RT-PCR.

Name of probe set	Fold change[a]	Description
Up-regulated genes		
rc-AA946443	12.1	Npdc1: Neural proliferation, differentiation and control, 1
rc-AA851204	11.5	Rat TNF receptor-1 associated protein (TRADD) mRNA, 3' end of cds
rc-AI010116	10.5	Rat cofactor required for Spl transcriptional activation, subunit 6(CRSP6) mRNA
rc-AA925183	8.8	Mouse homologue mRNA for 20 S proteasome non-ATPase subunit
rc-AI008106	8.1	Rat T clonel5 mRNA. mRNA for rat calcyclin
rc-AA850378	5.9	Human homologue methyl-CpG binding domain protein2 (MBD2) mRNA
rc-AI009350	4.8	Abhydrolase domain containing 4 (predicted) Rn. 1831
rc-AA925864	4.4	Rat aldose-reductase-like protein MVDP/AKR1B7 mRNA, complete cds
rc-AA899498	2.6	Catechol-O-methyltransferase domain containing 1 (predicted)
Down-regulated genes		
rc-AI029795	-7.7	Rat growth response protein (CL-6), complete cds
rc-AA924301	-6.2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2 (Abcc2)
rc-AI964628	-6.1	Rat mRNA for glucose-6-phosphatase catalytic subunit, complete cds
rc-AI044453	-4.9	Strongly similar to XP_544438.2 Predicted: similar to 1810010N17Rik protein
rc-AI029455	-4.2	Ttpa: tocopherol (alpha) transfer protein
rc-AI010568	-4.2	Ghr: Growth hormone receptor
rc-AI045040	-3.9	Dgat2: Diacylglycerol O-acyltransferase homolog 2 (mouse)
rc-AI045066	-3.5	Human homologue histidine-rich glycoprotein (HRG) mRNA

[a]Fold change column indicates results from the oligonucleotide array.

the array, we detected alterations in its expression in the livers of LEC rats under different levels of oxidative stress. Another 4 known oxidative stress-responsive genes, including G6PDH (18), thioredoxin (Trx) (19-20), HO-1 (21-22), and *Akr1a1* (23-25) were also analyzed. A summary of the relative expression levels of the genes detected by RT-PCR is shown in Fig. 3. The induction of *Akr1b7* in the livers of LEC rats aged 24 and 48 weeks was 486- and 27.5-fold higher, respectively, than that of age-matched LEA rats. In contrast, the expression of well-known oxidative stress genes including *G6PDH, Trx, HO-1*, and *Akr1a1* in LEC rat liver was only 1.2- to 2.3-fold higher than that of age-matched LEA rats. Consistent with the high level of oxidative stress observed, the expression of these 5 genes in LEC rats was higher than that of 24-week-old LEA rats.

Since real-time PCR is a more quantitative, specific, sensitive, and reliable method for detecting the expression of target genes at the RNA level when compared with traditional 'end-point' measurements of PCR products, we performed real-time PCR for *Akr1b7*. The results are shown in Fig. 4. The expression of *Akr1b7* in livers of LEC rats aged 24 weeks was 85-fold higher than that of age-matched LEA rats, and 16-fold higher than that of LEC rats aged 48 weeks old.

Transcriptional activity of nuclear factor kappa B (NF-κB). Several conserved sequence motifs throughout the promoter were found to be correlated with potential sites for the binding of transcription regulatory factors, as determined by computational analysis of ~6000-bp DNA sequences of the 5'-flanking region of rat *Akr1b7* using the TRANSFAC database (Fig. 5A). The key binding domains associated with oxidative stress were identified. The nuclear factor kappa B (NF-κB) sites (26-27) were located at position -389/398, -650/660, -3758/-3769, and -5657/-5667. The ARE/EpRE (28-29) sites were located at -1187/-1203, -2416/-2432, -2709/-2725, and -4226/-4242 (Fig. 5B).

To examine whether NF-κB is induced in livers of LEC rats in response to oxidative stress and serves as a potential mediator of the *Akr1b7* gene, we determined the binding activity of NF-κB in hepatic nuclear extracts from both strains by gel mobility shift assay using NF-κB binding consensus oligonucleotide. As shown in Fig. 6, NF-κB binding was detected as two bands, designated upper and lower to indicate slower and faster mobility through the gel, respectively. A faster migrating band was detected in both LEA and LEC rats. However, the marked increase in the amount of a slow migrating complex was observed in LEC rats. Previous studies have shown that the complex of slower and faster mobility observed in the nuclear extract from rat liver is a p65/p50 heterodimer and a p50/p50 homodimer, respectively (30). Since the activation of the p50/p60 heterodimeric form of NF-κB is particularly sensitive to oxidative stress and changes

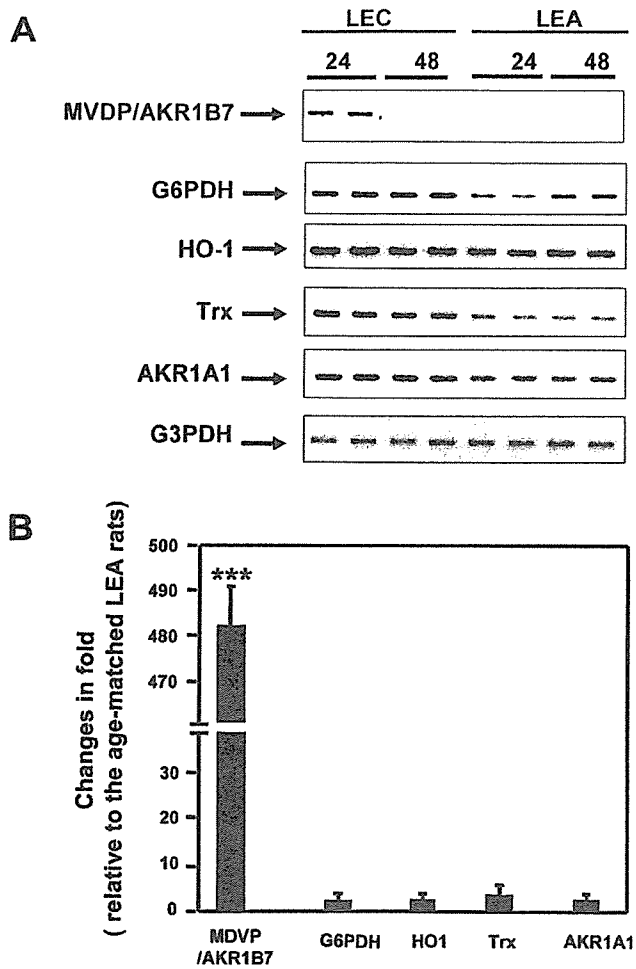

Figure 3. Summary of representative gene expression detected by RT-PCR. A, expression of MVDP/AKR1B7 (*Akr1b7*), G6PDH, HO-1, thioredoxin (Trx), AKR1A1, and G3PDH in the livers of LEC and LEA rats aged 24 and 48 weeks. B, relative expression of MVDP/AKR1B7, G6PDH, HO-1, TRX, AKR1A1, and G3PDH in the liver of LEC rats as compared with 24-week-old LEA rats. Values are means ± SD for 6 pairs of age-matched rats. ***Significantly different from the corresponding control group (*p<0.001).

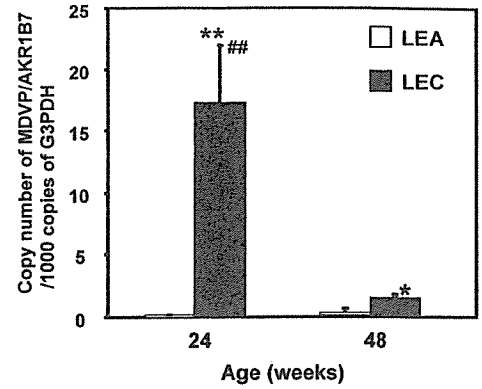

Figure 4. Expression of Akr1b7 quantitatively detected by real-time RT-PCR in livers of LEA and LEC rats at different ages. All values are expressed as mean ± SD of 4 animals. Student's t-test was performed for comparison of *Akr1b7* expression. * and **, significant difference at p<0.05, p<0.01 when compared with age-matched LEA rat group. ##Significant difference at p<0.01 when compared with 48-week-old LEC rats.

in cellular redox conditions (31-32), its observed up-regulation is consistent with a heightened state of oxidative stress in LEC rat liver.

To explore the possibility that NF-κB or cupric ion regulate transcriptional activity of 5'-upstream regions of *Akr1b7*, we investigated their effects on AKR reporter plasmids in transfection assays with HepG2 cells (Fig. 7). The reporter plasmid p6.0-AKR-Luc contains the upstream 6-kb DNA sequence of Akr1b7. To verify that p6.0-AKR-Luc shows transcriptional activity, reporter activities of the plasmids lacking or with the promoter were determined (Fig. 7A). When I-κB expression plasmid was co-transfected with p6.0-AKR-Luc in HepG2 cells, I-κB repressed its expression dose-dependently (Fig. 7B). In contrast, treatment with copper sulfate did not affect the transcriptional activity of p6.0-AKR-Luc, suggesting that it is unlikely that copper mediates transcriptional activity of the 6-kb sequence upstream of *Akr1b7* (Fig. 7C). In contrast with the transcriptional activity of ARE to redox-active agents, the transcriptional activity of p6.0-AKR-Luc was not induced by tBHQ (Fig. 7D). The ARE-reporter gene however was induced by tBHQ treatment (Fig. 7E).

Discussion

In our study, high levels of expression of MT (I/II), Hsp73, and other indicators of oxidative stress suggest that LEC rats at approximately 24 weeks of age undergo high oxidative stress levels. Furthermore, the high number of GST-P$^+$ single cells also suggested an increase in the number of preneoplastic cells in LEC rats at around 24 weeks of age. Moore and colleagues (33) reported that single GST-P$^+$ liver cells are a putative marker of initiated hepatocytes that can be useful for analyzing the initiation stage of carcinogenesis (34). Oligonucleotide array analysis and RT-PCR of liver tissue from 24-week old rats identified 17 transcripts that were differentially expressed in *Atp7b* m/m rats, a genotype generated by backcrossing LEC rats. Of the 17 differentially expressed genes, the expression of *Akr1b7* gene was most altered in LEC rats as well as LEA rats.

To compare the effects of oxidative stress on akr1b7 with those on other well-known oxidative stress-related genes, we selected G6PDH, HO1, Trx and Akr1a1, (which are sensitive biomarkers of oxidative-stress) for RT-PCR and measurement of hepatic mRNA expression in 24- and 48-week-old LEC and LEA rats. HO1 plays an important functional role as an endogenous antioxidant (21,22). Trx in conjunction with thioredoxin reductase, is a ubiquitous oxido-reductase system, positioned at the core of cellular thiol redox control and antioxidant defense (19). There is emerging evidence that the Trx system is as important as the glutathione system for cellular redox regulation of oxidative stress (20,35). MT and Trx proteins belong to the same family of thiol-rich compounds (36), and play important roles in the defense against reactive oxygen species (32). G6PDH can be up-regulated by hepatocellular oxidative stress (18), and AKR1A1 is responsible for the reduction of aldehydes, which are the main byproducts of lipid peroxidation (23,24). Interestingly, *Akr1b7* was the gene whose expression was up-regulated most in response to oxidative stress in LEC rat liver in the present study.

A

Name	Consensus sequence
NF-kappa B	GATAAG GGGAATTTTC
ARE/EpRE	$T^{A}_{C}Ann^{A}_{G}TGA^{C}_{T}nnnGC^{AA}_{GT}$

B

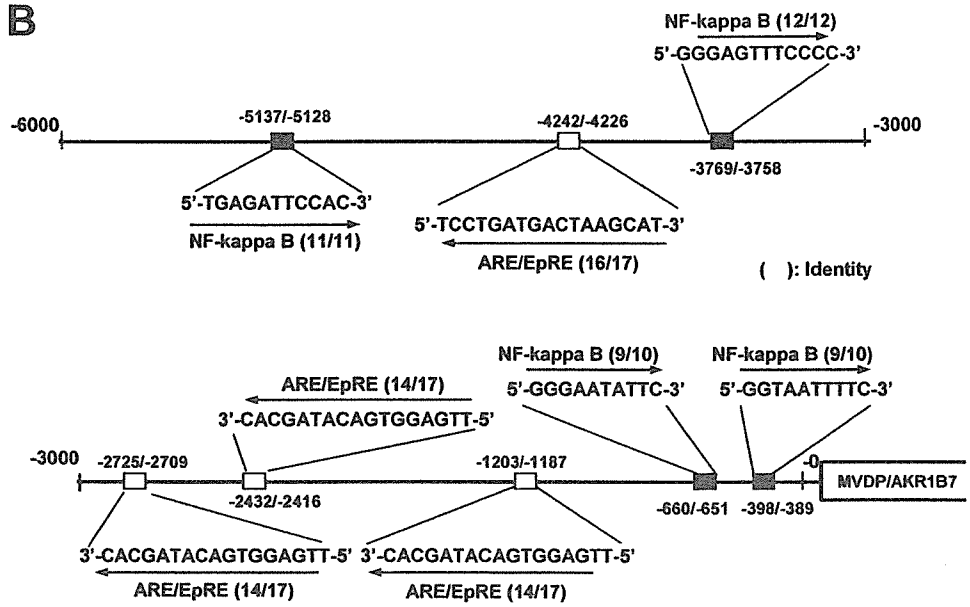

Figure 5. Genome informative analysis of binding motifs of the 5'-upstream regions of *Akr1b7* genes. A, typical genes having the NF-κB site or EpRE/ARE sequences. B, distributions of the NF-κB site and EpRE/ARE sequences are located 6 kb upstream of the *Akr1b7* gene.

AKR1B7 encodes an aldose-reductase involved in detoxification processes (37). It is the major enzyme responsible for the reduction of isocaproaldehyde, which is formed from the side-chain cleavage of cholesterol in the first step of murine steroidogenesis (38). Transcriptional regulation of *Akr1b7* has been well defined in two of the major organs in which it is expressed. In mouse adrenal cortex, its expression is acutely regulated by ACTH (39), whereas in the mouse vas deferens, a strong androgen-dependent accumulation has been described (40). Lau *et al* have shown by RNase protection that *Akr1b7* is also expressed in mouse kidney, eye, intestine and, to a very low extent, in liver (41). The present study is the first to report that *Akr1b7* is induced by copper overload in LEC rat liver.

Lefrançois-Martinez *et al* have shown that AKR1B7 can detoxify 4-hydroxynonenal (4-HNE), a cytotoxic, ß-unsaturated acyl aldehyde (38). This product is a natural result of the lipid peroxidation and cleavage that occurs in response to oxidative stress and aging (42). The previous studies showed that 4-HNE levels increased during the onset of hepatitis and development of hepatic tumors in LEC rats (43). Hence, we hypothesized that AKR1B7 could be one of several enzymes involved in the detoxification of lipid peroxides in the livers of LEC rats.

We also determined the expression of *Akr1a1*, which is ubiquitously localized and activates trans-dihydrodiols by

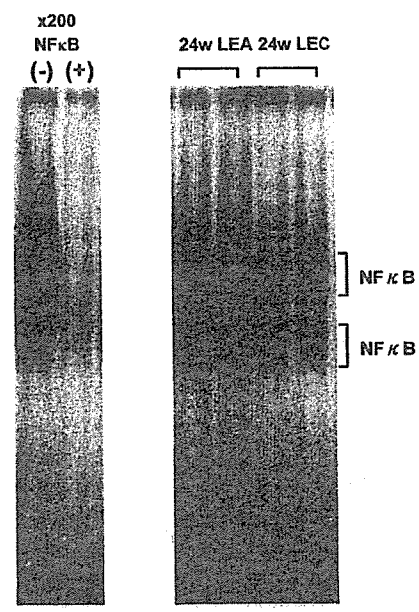

Figure 6. Involvement of transcription factor NF-κB in the transcriptional regulation of the MVDP/AKR1B7 gene. NF-κB is activated by hepatitis onset in LEC rat liver. NF-κB oligonucleotide binding activity in nuclear extracts prepared from the livers of 24-week-old LEA and LEC rats was determined by gel mobility shift assay using ^{32}P-labeled oligonucleotide as described under Materials and methods. The specificity of the NF-κB complex was verified by displacement with excess (250-fold) unlabeled oligonucleotide for NF-κB.

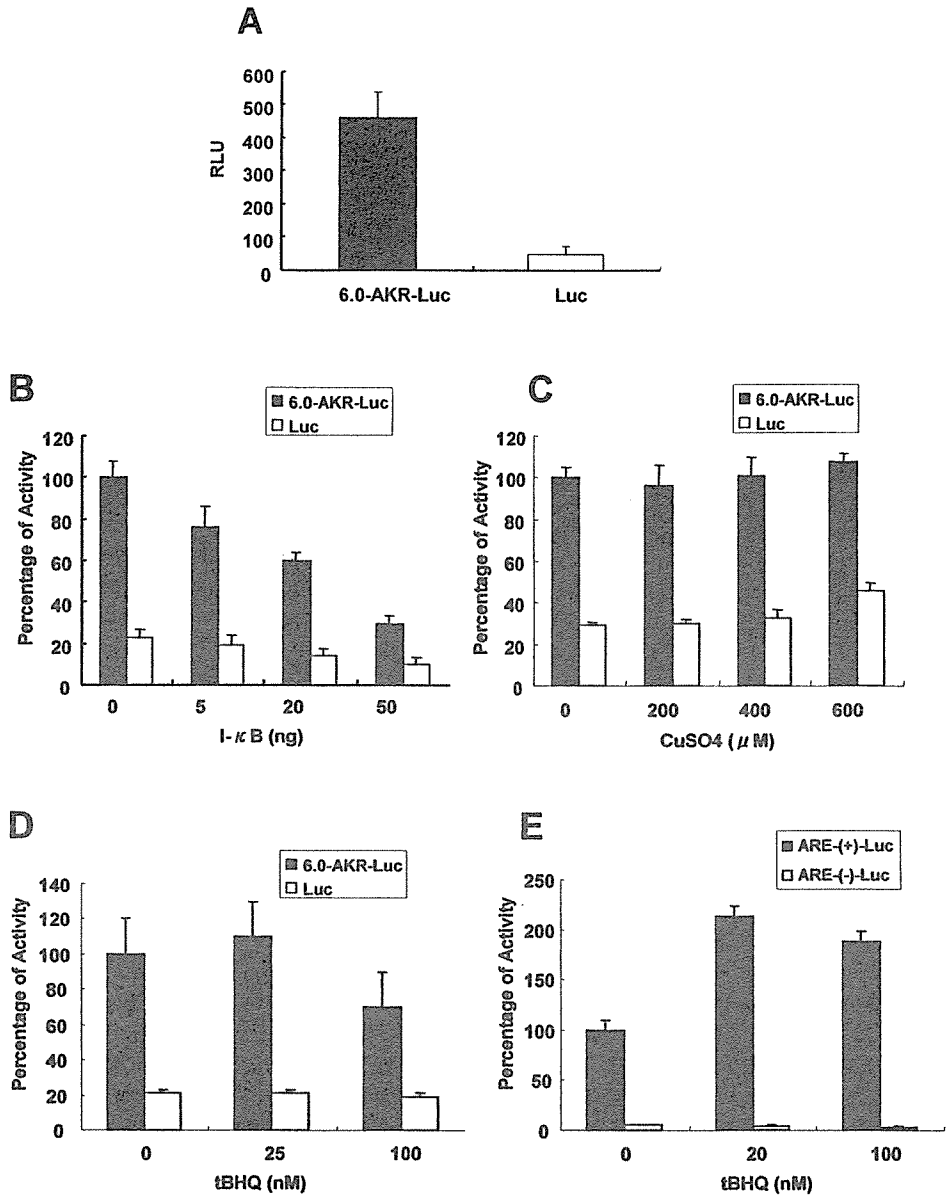

Figure 7. Effects of I-κB and copper on AKR promoter reporter luciferase activity. HepG2 cells were transfected with 6.0-AKR-Luc or Luc (100 ng/well, in a 24-well plate) reporter construct with internal control reporter Renilla luciferase (pRL-TK, 2 ng/well) for 24 h. Cells were collected 24 h after transfection and the firefly and Renilla luciferase activities measured using a Dual-luciferase reporter assay system. Firefly luciferase reporter activities were normalized to Renilla luciferase activities and expressed as relative light units (RLUs). A, AKR promoter reporter 6.0-AKR-Luciferase has activity in HepG2 cells. B, I-κB overexpression represses AKR promoter reporter 6.0-AKR-Luciferase activity in HepG2 cells. HepG2 cells were transfected with 6.0-AKR-Luc or Luc with various amounts of I-κB expression plasmids for 24 h. The RLU of the 6.0-AKR-Luc without I-kB overexpression was set as 100%. C, AKR promoter reporter 6.0-AKR-Luciferase did not respond to $CuSO_4$ in HepG2 cells. The RLU of the 6.0-AKR-Luc without $CuSO_4$ treatment was set as 100%. D, effects of tBHQ on AKR promoter reporter 6.0-AKR-Luciferase in HepG2 cells. The RLU of the 6.0-AKR-Luc without tBHQ treatment was set as 100%. E, effects of tBHQ on ARE(+)promoter reporter ARE(+)-Luciferase in HepG2 cells. The RLU of the ARE(+)-Luc without tBHQ treatment was set as 100%.

converting them to reactive and redox-active o-quinones (44); however it was not induced in the livers of LEC rats. Therefore, it is likely that AKR1B7 is primarily responsible for detoxification of specific oxidized molecules such as 4-HNE and etheno adenine, which are produced in LEC rat liver. Further studies should be conducted to confirm this possibility.

Since a search for binding motifs revealed 4 NF-κB binding sites in the 5'-upstream regions of the *Akr1b7* gene (Fig. 5), our results suggest that overexpression of the *Akr1b7* gene might be regulated partly through the NF-κB pathway. NF-κB is activated in response to a variety of stimuli, most of which are relevant to oxidative stress conditions (e.g., UV light, virus infections, H_2O_2, heat shock, phorbol esters, cytokines, etc.) (45). Furthermore, these oxidative stimuli cause a preferential increase in the p50/p65 heterodimeric form of NF-κB over the p50/p50 homodimer (31,45). As shown in Fig. 6, the nuclear level of the p50/p60 heterodimeric form of NF-κB was markedly increased in the livers of LEC rats as compared with LEA rats. These results suggest that NF-κB is activated by oxidative stress during the development of chronic hepatitis and hepatocellular carcinoma and may play a significant role in the induction of the *Akr1b7* gene in LEC rat liver.

Another interesting observation in this study was that electrophile response element (EpRE), RGCNNN(C/G)TCA (28,46), and NF-κB were located at several sites in the 5'-upstream regions of *Akr1b7* (26), as shown in Fig. 5. The information about these response elements suggests that expression of *Akr1b7*, as well as the GST-P gene, reflects oxidative stress from copper overload, such as that which occurs in the LEC rat model used in the present study, as well as the GST-P gene. Therefore, a transient transfection assay using the 6-kb upstream of *Akr1b7* fragment (p6.0 kb-AKR-Luc) was conducted. Since NF-κB is activated constitutively in HepG2 cell lines (47), IκB inhibited luciferase activity induced by p6.0 kb-AKR-luc even in unstimulated HepG2 cells (Fig. 7), indicating that transcriptional activity of *Akr1b7* is involved in the NF-κB pathway. In contrast, cuprous ions did not induce the luciferase activity of p6.0 kb-AKR-Luc, suggesting that copper signaling may not directly affect its transcriptional activity. However, several studies demonstrated that cellular damage by copper mediates NF-κB signaling and that the copper chelating agent thiomolibdate suppresses NF-κB activation (48-51). Based on these studies and our present observations from the transfection assays, we speculate that the *Akr1b7* gene is mainly induced by NF-κB activation, which is mediated through copper accumulation in the hepatocytes of LEC rats.

In conclusion, oligonucleotide array analysis showed that oxidative stress induced by abnormal accumulation of copper can up- or down-regulate the expression of a battery of genes in LEC rat liver, which are mainly related to oxidative stress, growth regulation, and transcriptional regulation. We characterized gene expression changes that were specifically dependent on the oxidative stress level. Compared with well-known oxidative stress-responsive genes, the *Akr1b7* gene was highly expressed in LEC rats as a consequence of high oxidative stress levels, suggesting that it is involved in the defense against oxidants in the response to oxidative stress. Our study also demonstrated that overexpression of the *Akr1b7* gene might be regulated partly through the NF-κB pathway.

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Activation of telomerase in BeWo cells by estrogen and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in co-operation with c-Myc

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Abstract. Telomerase activation, known to be stimulated by estrogen, is essential for cellular immortalization and transformation, both of which play a role in tumorigenesis. Dioxin and dioxin-like compounds have been shown to induce endometriosis and promote estrogen-dependent tumors. In this study, we show that either 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or a combination of TCDD and 17-ß estradiol (E2) increase telomerase activity and the expression of the human telomerase catalytic subunit (hTERT) in human choriocarcinoma (BeWo) cells. Compared with estrogen or TCDD alone, the combination treatment did not show an additive effect. Likewise, treatment with either E2 or TCDD increased DNA synthesis and the cell population in S-phase, as detected by FACS analysis. However, following treatment with the E2 and TCDD combination, the proportion of cells in S-phase was actually lower than in cells treated with TCDD alone. These results suggest that TCDD alone mimics estrogenic action in telomerase activation and cell proliferation but, in the presence of estrogen, TCDD-induced actions were partially counteracted. E2 and TCDD also induced c-Myc, which is a transcriptional activator of hTERT in BeWo, but neither of these agents induced telomerase activity in HO15.19 *c-myc*-null cells. In contrast, only TCDD upregulated telomerase in TGR-1 cells, which are c-Myc expressing but lacking ER expression. The findings suggest that TCDD induces telomerase activity mediated through AhR signaling and/or ER-independent c-Myc signaling. The present study provides insight into the mechanism of promoter activity of TCDD in estrogen-related tumors.

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Key words: telomerase, TCDD\hTERT\c-Myc\estrogen\AhR\ARNT

Introduction

Exposure to the halogenated aromatic hydrocarbon, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), elicits a number of biological and toxicological responses which include both endocrine disruptive effects and cancer (1-3). In particular, recent animal experiments have demonstrated a number of female reproductive toxicities resulting from TCDD exposure, including induction of ovarian tumors in rats, placental hypoxia in pregnant rats and inhibition of estradiol secretion from human luteinizing granulosa cells (4-6). Most TCDD-induced toxic responses are thought to be mediated via binding to the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor and a member of the basic helix-loop-helix/PER-ARNT (aryl hydrocarbon nuclear translocator protein)-SIM family of proteins (7). In addition, the mechanism of action of TCDD is similar to that proposed for steroid hormones (8,9). Ligand-bound AhR heterodimerizes with ARNT and this complex then binds to specific gene regulatory sequences, known as xenobiotic or dioxin responsive elements (XRE or DRE). The induction of *CYP1A1* gene expression by TCDD is considered to be an early response signal and also the most sensitive biomarker for assessment of exposure and sensitivity levels to this compound (10,11).

TCDD also disrupts endocrine-responsive pathways, including those involving steroid hormone metabolism (8,12). It has also been previously demonstrated that CYP1A1 induction is influenced by the estrogen receptor (ER) and that a functional ER is required for this activation (13-15). In more recent studies, it was demonstrated that the presence of 17-ß estradiol (E2) in ovariectomized rats could significantly influence the induction of CYP1A1 by TCDD (16). These studies indicated that the presence of ER in the nucleus was an absolute requirement for the TCDD-mediated response of CYP1A1. Recently it has been demonstrated that the estrogenic action of AhR agonists, such as TCDD, may be activated through the formation of a functional transcriptional complex by the interaction of AhR/ARNT and unliganded ER, which then targets estrogen response element (ERE)-like regions (17). Other studies have previously shown differential expression of AhR and ARNT mRNA throughout the estrous cycle in rodents (18), suggesting that this expression is involved in estrogen and/or ER regulation in this system. Each of these findings indicates that both E2 and ER have significant effects upon TCDD-liganded AhR signaling, which can lead to the

induction of tumorigenesis. Collectively, these studies also implicate both AhR and ER signaling in the cross-talk between a number of responses induced by both of these receptors. However, the molecular mechanism(s) underlying the development of malignant cells via an estrogen response, following AhR signaling, is still unknown. One of the new emerging functions of ER is the upregulation of human telomerase activity (19). Telomerase is a cellular reverse transcriptase that catalyzes the synthesis and extension of telomeric DNA repeats (20,21). In most normal human somatic cells the level of telomerase activity is extremely low or undetectable. It has been shown in cell culture that telomeres progressively shorten after each cell division until critically short lengths signal cellular senescence (22,23). However, a variety of cell lines and malignant tumors have been found to express high levels of telomerase activity (24-26). This suggested that telomerase activation may be a critical step in cell immortalization and oncogenesis. One of the key components of human telomerase is its catalytic subunit, hTERT, which is actively upregulated by c-Myc (27). hTERT is also transcriptionally activated by ER, which binds to the ERE present in the upstream hTERT promoter region and induces increased expression of the telomerase enzyme (19). This activation of telomerase by E2 may partly explain the carcinogenic properties of estrogen. There are no reports in the literature, however, that describe how TCDD impacts upon telomerase gene expression in cells regulated by estrogen. Nevertheless, a previous report has shown that TCDD did not affect telomerase activity in either normal human epidermal cells, immortalized cells or malignant keratinocytes (28). The human choriocarcinoma BeWo cell line expresses a functional ER that is important for the control of BeWo cell proliferation (29), and highly produces E2 (30). These characteristics in BeWo are useful for the study of interactions between E2 and TCDD in female reproductive organs.

In the present study, we have examined the effects of E2, TCDD, and the two compounds in combination on telomerase activity in BeWo cells and attempted to elucidate the mechanisms underlying the interaction of estrogen and TCDD and the subsequent activation of hTERT mediated through c-Myc.

Materials and methods

Cell culture and treatment. The BeWo (human choriocarcinoma) cell line was obtained from the American type culture collection (Rockville, MD) and routinely maintained in DMEM/Ham's F12 (1:1) medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 units penicillin/ml and 100 μg streptomycin/ml) under standard conditions in a 37°C incubator with 5% CO_2. TGR-1 and HO15.19 rat fibroblast cells, which are c-Myc expressing and *c-myc* null (referred to here as Myc+/+ and Myc-/-, respectively), were a generous gift from Dr M. Cole (University of Princeton, NJ). These cells were maintained in DMEM F12 medium supplemented with 10% FBS. All tissue culture reagents were purchased from Invitrogen (Carlsland, CA). The cells were maintained by refreshing the medium twice a week and by passaging once. For each of the induction assays, cells were grown in phenol red-free media containing 10% charcoal-stripped serum for 48 h prior to treatment.

Stretch polymerase chain reaction (PCR) assay. For quantitative analysis of telomerase activity, stretch PCR assays were performed using the Telochaser system according to the manufacturer's instructions (Toyobo, Tokyo, Japan). This method was developed originally by Tatematsu *et al* (31). The PCR products were electrophoretically separated using a GenePhor electrophoresis system (Amersham Biosciences, Tokyo) unit and visualized using Sybr-Green I (FMC Bioproducts, Rockland, ME). To ensure the efficiency of PCR amplification, 10 ng of internal control phage DNA sequence (Toyobo) was added to each PCR reaction. The relative telomerase activity was determined by measuring the band intensities of the telomerase ladders and comparing them with internal standards using a Molecular Imager and its software, Quality One (BioRad, Hercules, CA).

Real-time PCR and RT-PCR. hTERT mRNA expression was analyzed by real time PCR according to the method of Bieche *et al* (32). Briefly, the primer sequences for hTERT amplification were 5'-TGACACCTCACCTCACCCAC-3' and 5'-CACTGTCTTCCGCAAGTTCAC-3', and the probe sequence was 5'-ACCCTGGTCCGAGGTGTCCCTGAG-3'. Ribosomal phosphoprotein P0 (RPLP0) was used as the internal control and the PCR primer sequences were 5'-GGC GACCTGGAAGTCCAACT-3' and 5'CCATCAGCACCAC AGCCTTC3', whereas the probe sequence was 5'-ATCTGC TGCATCTGCTTGGAGCCCA-3'. cDNA was synthesized from 1 μg of total RNA in the presence of 1.5 μM random hexamers and 50 units of superscript II RNase H-reverse transcriptase (Life Technologies, Carlsland, CA). For each PCR run, the master mix contained 150-nM of probe, 300-nM of each primer and 10 μl of each diluted c-DNA in a final volume of 50 μl. Thermal cycling was performed using an ABI PRISM 7000 PCR machine (Applied Biosystems, Foster City, CA) with the following program: 95°C for 10 min, 50 cycles at 95°C for 15 sec, 65°C for 1 min. Reverse transcriptase (RT)-PCR analysis of CYP1A1, AhR and ARNT was performed as previously described (13). Briefly, total RNA was isolated using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol and cDNA was synthesized from 1 ug of RNA using a Revertrace RT-PCR kit (Toyobo) with $OligoDT_{20}$. The efficiency of cDNA synthesis from each sample was estimated by PCR with glyceraldehyde-3-phosphate dehydrogenase-specific primers as the internal control and also performed as described previously (13).

Cell-cycle analysis. BeWo cells were starved of serum for 24 h and then stimulated with charcoal-stripped serum containing either E2, TCDD or both at the indicated concentrations. After 24h, the cells were trypsinized and washed in chilled phosphate-buffered saline (PBS), fixed in 70% ethanol, digested with RNase (0.02 μg/μl) and stained with 50 μg/ml propidium iodide. The cells were sorted and analyzed using a Becton-Dickinson FACScan (BD Biosciences, SanJose, CA) and ModFit LT software (Topsham, ME).

Western blotting. BeWo cells were treated with either E2, TCDD, or both, for 24 h. Then, to prepare cell extracts, cells were washed three times with PBS and lyzed for 30 min on ice