EXPRESSION AND REGULATION OF HUMAN XANTHINE OXIDOREDUCTASE

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ACADEMIC DISSERTATION

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The truth is rarely pure and never simple.

Oscar Wilde, *The Importance of Being Earnest, 1895*

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals:

I. Annikki Sarnesto, **Nina Linder** and Kari O. Raivio. Organ distribution and molecular forms of human xanthine dehydrogenase/xanthine oxidase protein.
Lab Invest 1996; 74: 48-56.

II. **Nina Linder**, Juhani Rapola, and Kari O. Raivio. Cellular expression of human xanthine oxidoreductase protein in normal human tissues.
Lab Invest 1999; 79: 967-974.

III. **Nina Linder**, Eeva Rytkönen, Risto Lapatto, and Kari O. Raivio. Post-translational inactivation of human xanthine oxidoreductase in normal cell-culture conditions.
Am J Physiol Cell Physiol 2003 Jul; 285(1):C48-55.

IV. **Nina Linder**, Johan Lundin, Jorma Isola, Mikael Lundin, Kari O. Raivio, and Heikki Joensuu. Downregulated xanthine oxidoreductase is a feature of aggressive breast cancer. Clin Cancer Res. In press.

ABBREVIATIONS

AO	aldehyde oxidase
BEAS-2B	human bronchial epithelial cells
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
COX-2	cyclooxygenase-2
dATP	deoxy ATP
dGTP	deoxy GTP
DCPIP	dichlorophenolindophenol
DDFS	distant disease free survival
ERBB2	avian erythroblastic leukemia viral oncogene homolog
FAD	flavine adenine dinucleotide
HER-2	human epidermal growth factor receptor 2
HIF-1	hypoxia-inducible factor-1
HPRT	hypoxanthine phosphoribosyltransferase
HPLC	high performance liquid chromatography
HRE	hypoxia responsive element
IMP	inosine monophosphate
I/R	ischemia-reperfusion
mRNA	messenger ribonucleic acid
PBS	phosphate-buffered saline
p53	tumor suppressor protein p53
RPA	ribonuclease protection assay
RR	risk ratio
RT-PCR	reverse transcriptase-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	tris buffered saline
TTBS	Tween 20 in tris buffered saline
XDH	xanthine dehydrogenase
XO	xanthine oxidase
XOR	xanthine oxidoreductase

ABSTRACT

Xanthine oxidoreductase (XOR) is a complex molybdoflavoprotein that is the terminal enzyme of purine catabolism in man and higher apes. XOR has been extensively studied in a number of species, but human studies are few. Because of its metabolic position and the characteristics of the 5´-regulatory region of its gene, and because it has been implicated in human pathology, we also hypothesized that the regulation of XOR expression in man is unique and cannot be extrapolated from animal studies. Since XOR is expressed in the normal and lactating mammary gland, and because animal studies have suggested an involvement of XOR in tumorigenesis, we hypothesized that XOR may be differentially expressed in cancerous human mammary epithelial cells.

We purified the enzyme from human milk and produced anti-XOR antibodies in order to study the molecular forms, the ratio of XOR activity to protein, and the cellular localization of XOR in normal human tissues. The results showed that human XOR has a molecular size similar to other mammalian XOR enzymes. Most of the enzyme is in an inactive form in milk but not in liver and intestine, in which the relationship between XOR activity and protein is constant during ontogenesis. In normal human tissues studied, the protein is localized to periportal hepatocytes and epithelial cells of the mammary gland and intestine. The vascular endothelium of the intestine, mammary gland, and skeletal muscle also express XOR, whereas no cells in the heart, brain, or lung show immunoreactivity.

Given that oxygen is a substrate of XOR and hypoxia has been shown to regulate XOR in cultured animal cells, we investigated the regulation of the protein in cultured human bronchial epithelial cells under different oxygen concentrations. XOR activity is increased in hypoxia with no change in XOR protein concentration, mRNA expression or promoter activity, whereas in hyperoxia the enzyme is inactivated with no change in protein or mRNA levels. Indirect evidence suggested that the mechanism of inactivation by oxygen, already under normal cell culture atmosphere, occurs at the molybdenum center of XOR, and that the inactivation is reversed in hypoxia, resulting in apparent induction of enzyme activity.

Using tissue microarray samples from a large population-based cohort of breast cancer, with well-characterized clinicopathological parameters and outcome data, we demonstrated that XOR is downregulated in more than half of the breast tumors studied, and that absence of XOR is an independent predictor of unfavorable outcome.

In conclusion, this series of studies clarified the distribution of XOR in normal human tissues and the mechanisms of regulation of the enzyme by oxygen, thus providing a better understanding of the physiological behavior of the enzyme. Moreover, the differential expression of XOR in breast cancer may have clinical implications as a novel prognostic marker.

1. Pathways of human purine metabolism

The major purine compounds in the cell are adenine and guanine ribo- and deoxyribo-nucleotides and nucleic acids. They play an essential role in energy-requiring reactions, nucleic acid synthesis, and as signaling molecules.

Net contributions to body pools of purine compounds are by synthesis of purine nucleotides from nonpurine precursors (*de novo* synthesis) or by dietary purine ingestion. Since the energy cost of synthesizing a purine nucleotide *de novo* is six times higher, in ATP equivalents, than that of reutilizing an intact purine ring, cells have efficient mechanisms for the salvage of degraded purines (Raivio et al., 2001).

Hypoxanthine is quantitatively the most important purine catabolic product, since the breakdown pathways of adenine ribo- and deoxyribonucleotides converge to yield hypoxanthine. Its main metabolic fate is phosphoribosylation by hypoxanthine phosphoribosyltransferase (HPRT) to inosine monophosphate (IMP), i.e. the salvage pathway. Yet, in situations of increased nucleic acid or ATP degradation, the purine catabolic pathway is overloaded and degradation products accumulate (Raivio et al., 2001).

Xanthine oxidoreductase (XOR) catalyzes the two last steps in the catabolic pathway in humans and higher apes, with uric acid as the end product of purine metabolism, whereas in all other animal species uric acid is further metabolized into allantoin by uricase. Guanine nucleotides are catabolized to guanine, which is a substrate of HPRT and can thus be reutilized, or it may be deaminated to xanthine and then oxidized by XOR to uric acid (Figure 1)

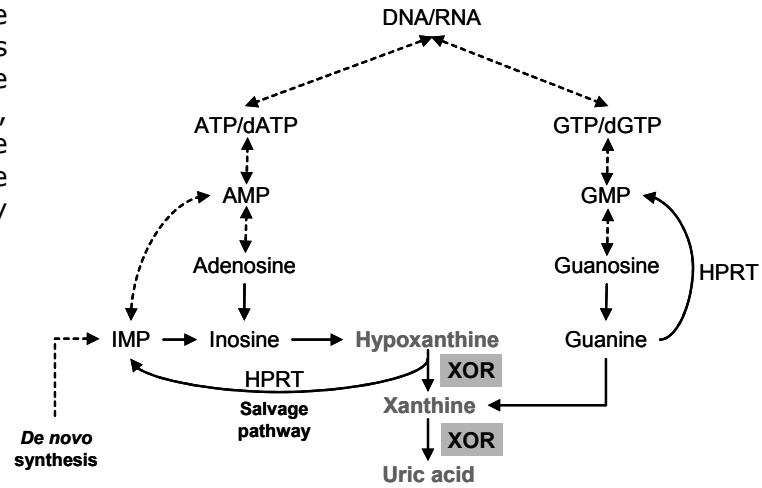

Figure 1. Pathways of human purine ribonucleotide metabolism. The reactions catalyzed by XOR and the purine salvage pathway are demonstrated. XOR, xanthine oxidoreductase; IMP, inosine monophosphate; HPRT, hypoxanthine phosphoribosyltransferase; dATP, deoxy ATP; dGTP, deoxy GTP.

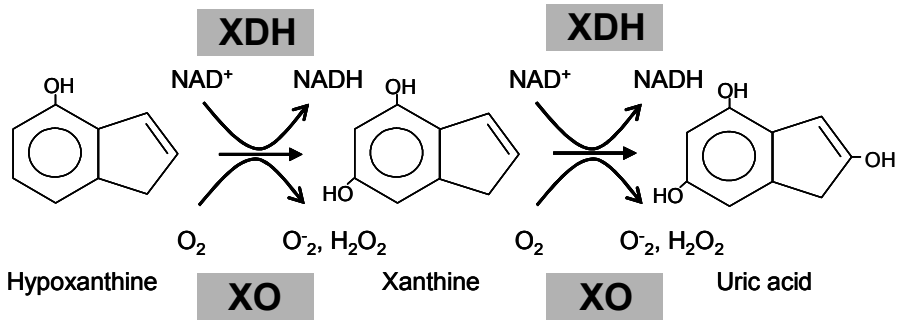

Figure 2. Reactions catalyzed by the dehydrogenase (XDH) and oxidase (XO) forms of xanthine oxidoreductase. O_2.-, superoxide; H_2O_2, hydrogen peroxide.

2. Biochemistry of xanthine oxidoreductase

2.1. Basic reactions catalyzed by XOR

Xanthine oxidoreductase (XOR) oxidizes hypoxanthine to xanthine and xanthine to uric acid (Figure 2). The enzyme exists in two forms, xanthine dehydrogenase (XDH, EC 1.1.1.204) and xanthine oxidase (XO, EC 1.1.3.22). Both enzymes can reduce molecular oxygen to superoxide anion and hydrogen peroxide, but the preferred electron acceptor is NAD^+ for XDH and molecular oxygen for XO (Hille and Nishino, 1995). Other purine substrates of XOR include adenine (Krenitsky et al., 1986), a number of synthetic purine derivatives, e.g. 6-mercaptopurine (Krenitsky et al., 1986), and allopurinol (Massey et al., 1970).

The reactive oxygen species, which include superoxide and hydrogen peroxide, produced by XO react with and modify the structure and function of macromolecules including proteins, lipids, carbohydrates, and nucleic acids (Castro and Freeman, 2001). Reactive oxygen species can also serve as second messengers in signal transduction and participate in cellular functions such as cytokine and growth factor action, ion transport, and regulation of transcription (Lander, 1997). On the other hand, the primary product of XOR, uric acid, is an antioxidant and has been ascribed a protective role against free radical effects (Ames et al., 1981).

2.2. Structure of XOR protein

XOR and aldehyde oxidase (AO) utilize a molybdenum cofactor and a flavin to oxidize their respective substrates, and these enzymes are the only members of the family of molybdoflavoproteins in mammals (Garattini et al., 2003). The two enzymes have remarkable similarities. They have a high level of amino acid identity, as well as similar molecular weights (Ichida et al., 1993; Terao et al., 2000), and a high degree of conservation of the intron/exon junctions (Terao et al., 1998), suggesting a common evolutionary origin.

The purified XOR is a homodimer, consisting of identical subunits of ~150 kDa each containing domains with molecular masses of 20, 40, and 85 kDa (Hille and Nishino, 1995). Each subunit includes one

molybdopterin cofactor, two non-identical iron-sulfur (Fe/S) clusters, and one flavin adenine dinucleotide (FAD) center (Figure 3), and the subunits are connected to each other with a linker peptide. The molybdenum in the molybdenum cofactor is covalently linked to two essential cysteins and to one essential sulfur atom (Enroth et al., 2000).

The oxidation of hypoxanthine and xanthine takes place at the molybdopterin site, where the purine substrate is bound to the molybdenum and donates two electrons, reducing Mo (VI) to Mo (IV) (Hille and Nishino, 1995). The arrangements of the cofactors indicate that the Fe/S clusters provide an electron transfer pathway from the molybdopterin cofactor to FAD (Enroth et al., 2000; Hille and Nishino, 1995). In addition, it has been suggested that the Fe/S clusters have a role as an electron sink to provide electrons to the FAD cofactor (Nishino and Okamoto, 2000). Finally, the reduction of the co-substrates NAD^+ or molecular oxygen occurs at the FAD site (Hille and Nishino, 1995) (Figure 3).

2.3. Dehydrogenase to oxidase conversion

XOR is synthesized as the dehydrogenase (XDH) form, which appears to be the predominant enzyme type in intact and freshly prepared mammalian tissue (Stirpe and Della Corte, 1969). However, the dehydrogenase (XDH) can be converted to the oxidase (XO), reversibly by sulfhydryl (SH) oxidation or irreversibly by proteolysis. Reversible XDH to XO conversion is due to SH-group oxidation of specific cysteine residues and generation of disulfide bridges (S-S), which can be reversed by SH-reducing agents, such as dithiothreitol (Nishino, 1997).

Treatment of XOR by trypsin nicks isolated rat liver XOR into three fragments of 20, 40, and 85 kDa, but the peptide fragments are not dissociated and XOR activity is retained. The fragments are separated by electrophoresis under denaturing conditions (Amaya et al., 1990). The crystal structure of bovine milk XOR has revealed that proteolytic cleavage of XDH leads to major structural alterations

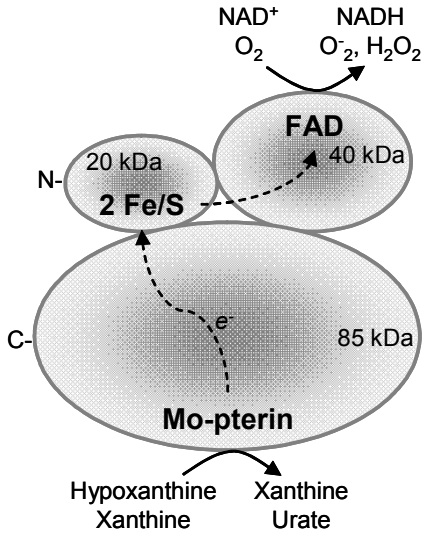

Figure 3. Proposed domain structure of XOR. Oxidation of xanthine and hypoxanthine occurs at the molybdopterin site (Mo-pterin), and reduction of NAD^+ or molecular oxygen takes place at the flavin adenine dinucleotide (FAD) site. The amino (N) and carboxy-terminal (C) ends of the protein are indicated.

around the FAD site and access of the NAD substrate to the FAD center is blocked (Enroth et al., 2000). By rapid purification, and the use of dithiothreitol, the enzyme from bovine milk remains in the XDH form (Hunt and Massey, 1992).

2.4. The human XOR gene

The human XOR gene was initially mapped to chromosome band 2p22 (Ichida et al., 1993; Rytkonen et al., 1995), but was later assigned to band 2p23 (International Human Genome Sequencing Consortium 2001). The nucleotide sequence has an open reading frame of 3999 nucleotides, corresponding to 1,332 amino acids. The primary structure of XOR is well conserved across the phylogenetic spectrum with high homology (~90 % identity) between human (Ichida et al., 1993; Saksela and Raivio, 1996), rat (Amaya et al., 1990), and mouse (Terao et al., 1992) enzymes. The human XOR gene spans at least 60 000 bp of DNA and is composed of 36 exons and 35 introns (Xu et al., 1996).

In contrast to the respective genes themselves, the 5´-untranslated regions of the XOR gene in human (Xu et al., 1996), rat (Chow et al., 1994) and mouse (Cazzaniga et al., 1994) are remarkably different, indicating different transcriptional regulation. The 5´-flanking region of the human XOR gene contains several consensus sequences relevant to inflammation. These include potential binding sites for interleukin-1 and -6, nuclear factor-κB, tumor necrosis factor-α, and interferon-γ . Sequences for binding of transcription factors involved in developmental regulation have also been found, including activator protein-1 and -2, and homeobox promoter sites (Xu et al., 1996). It has been shown that the promoter activity of the human XOR gene is repressed relative to the mouse gene. An E-box and a TATA-like element present in the human but not in the mouse promoter apparently are required for the control of human XOR promoter activity. Another difference is that the 5´-flanking region of the human XOR gene has a larger number of consensus sequences for potential promoter elements than do either the rat or the mouse (Xu et al., 2000). In addition, the transcription factor nuclear factor-Y is important for activating the human XOR gene (Martelin et al., 2000).

2.5. Inactivation of XOR

Inhibitors of XOR can be divided into two categories; molecules which are structural analogs to purine substrates and molecules which are unrelated to physiologic substrates. Allopurinol and its major metabolite oxypurinol are purine analogs that form a tight-binding complex with molybdenum at the active site, leading to the inhibition of XOR (Massey et al., 1970).

Inactive forms of XOR have been well documented. Loss of a sulfur atom from the active site results in inactivation of milk XOR (Nishino et al., 1983), and in rat liver *in vivo*, up to 50% of XOR has been shown to be inactive (Ikegami and Nishino, 1986). Cyanide is also capable of modifying the molybdopterin cofactor by removing the essential sulfur atom, thus inactivating the enzyme (Coughlan et al., 1980). This may also be the mechanism by which the enzyme is inactivated in hyperoxia by oxygen metabolites *in vivo* (Terada et al., 1988). Desulfo-XOR can be reactivated by reinsertion of the sulfur atom at the molybdenum site, which is catalyzed by a sulfurase present in human tissues (Ichida et al., 2001). Loss of a molybdenum atom or the pterin cofactor from the enzyme also results in inactivation of the enzyme (Ventom et al., 1988).

3. Expression of XOR in organs and cells

The wealth of biochemical information on isolated XOR is in contrast to the lack of knowledge on the cellular localization and the physiological function of this enzyme, especially in humans. XOR activity has been detected in almost all species studied (Parks

and Granger, 1986). In mammals, the liver and intestine have the highest XOR activity of any tissue (Al-Khalidi and Chaglassian, 1965). The liver is also a rich source of AO, whereas in the lung AO protein and mRNA are strongly expressed, while XOR expression is low. Conversely, the intestine is rich in XOR, while AO expression is low (Li Calzi et al., 1995; Saksela et al., 1998).

Substantial differences in organ distribution of XOR activity exist between mammalian species, making it difficult to extrapolate animal study results to human. The discrepancies in XOR expression between species may be explained by significant differences in the promoter regions (Xu et al., 1996) (Cazzaniga et al., 1994; Chow et al., 1994), suggesting diverse transcriptional regulation.

3.1. Non-human species

In one previous study bovine hepatocytes and intestinal epithelial cells lacked demonstrable XOR protein, whereas mammary gland epithelial cells and capillary endothelial cells of a variety of tissues including the mammary gland, liver, lung, skeletal muscle, heart, and intestine showed XOR immunoreactivity (Jarasch et al., 1981). In contrast, several other studies have localized XOR in the hepatocytes, e.g. in rat (Ichikawa et al., 1992) and chicken liver (Hattori, 1989). Data on XOR activity in the heart are also inconsistent, showing high XOR activity in the myocardium of the dog and rat (Chambers et al., 1985; de Jong et al., 1990), but low activity in the rabbit (Downey et al., 1988). Serum of some animals contains low XOR activities, whereas rat and mouse serum are relatively rich in XOR activity (Parks and Granger, 1986).

In the mouse, XOR mRNA is expressed in the mammary gland epithelium (McManaman et al., 2002), and in the liver, lung, and intestine (Kurosaki et al., 1995).

By immunoelectron microscopy of rat hepatocytes the subcellular localization of XOR was shown to be solely cytosolic (Ichikawa et al., 1992). However, in another study, XOR activity was identified by electron microscopy in peroxisomes, rough endoplasmic reticulum, lysosomes, and endocytic vesicles of rat hepatocytes (Frederiks and Vreeling-Sindelarova, 2002). Thus, the subcellular localization of XOR still remains controversial.

3.2. Human tissues

In general, XOR activity in human tissues is 10-100 times lower than in rodents, including rat and mice (Al-Khalidi and Chaglassian, 1965; Krenitsky et al., 1974; Wajner and Harkness, 1989). In humans, relatively high XOR activities are clearly present in the liver, intestine, mammary gland and milk (Al-Khalidi and Chaglassian, 1965; Hellsten-Westing, 1993; Kooij et al., 1992; Saksela et al., 1998) (Table 1). In contrast, data on XOR in serum, brain, and myocardium are conflicting, showing both measurable activity (Table 1) and lack of activity (Vettenranta and Raivio, 1990) (Al-Khalidi and Chaglassian, 1965; Parks and Granger, 1986). The subcellular localization of XOR in human is still unknown.

3.3. XOR expression in cell lines

Several non-human cell lines such as those originating from bovine (Terada et al., 1992b) and rat (Hassoun et al., 1994) endothelium, as well as mouse mammary epithelium (McManaman et al., 2000) show measurable XOR activity. Cultured human cells generally do not express XOR when cultured in normoxia (21% O_2) (unpublished data from our laboratory). However, an SV-40 immortalized human mammary epithelial cell line (HB4a) (Page et al., 1998) as well as isolated human aortic endothelial cells (Zweier et al., 1994) showed measurable XOR activity.

Table 1. Positive findings of XOR activity, protein, and mRNA in human.

XOR			Reference
Activity	**Protein**	**mRNA**	
Intestine, liver, lung	-	Liver, intestine, lung, kidney, heart, brain	(Saksela et al., 1998)
-	-	Liver, intestine	(Wright et al., 1995)
Liver, intestine	-	-	(Vettenranta and Raivio, 1990)
Liver, kidney, heart, brain	-	-	(Wajner and Harkness, 1989)
Liver, skeletal muscle, milk	Cardiac- and skeletal muscle cells, capillary endothelial cells of cardiac- and skeletal muscle, mast cells, macrophages	-	(Hellsten-Westing, 1993)
Liver (periportal and pericentral hepatocytes), enterocytes and goblet cells in jejunum	-	-	(Kooij et al., 1992)
-	Liver hepatocytes, bile duct epithelial cells	-	(Martin et al., 2004)
-	Liver hepatocytes and sinusoidal lining cells, mucosal cells of duodenum, arterial endothelial cells of duodenum heart, kidney, brain, and lung	-	(Moriwaki et al., 1993)
Milk	-		(Page et al., 1998)
-	Mammary gland epithelial cells	-	(Cook et al., 1997)
-	Perinuclear and surfaces that appose neighboring cells of mammary gland epithelial cells (HB4a), and HUVEC in culture	-	(Rouquette et al., 1998)
-	Myocytes in myometrium, villous stromal cells of the placenta, fetal membranes	-	(Telfer et al., 1997)
-	Serum	-	(Adachi et al., 1993; Battelli et al., 1999; Pesonen et al., 1998)
Heart	-	-	(de Jong et al., 1990; Eddy et al., 1987)

-, not analyzed; HUVEC, human umbilical vein endothelial cells; RT-PCR, reverse transcriptase-PCR.

4. Regulation of XOR

Despite the biochemical function of XOR as a housekeeping enzyme in the catabolism of purines, its activity can be regulated by a variety of stimuli.

4.1. Inflammatory mediators

Inflammatory mediators, particularly interferons, induce XOR in experimental models. Interferon-α and interferon inducers, such as lipopolysaccaride, increase XOR activity and mRNA concentrations *in vivo* in various mouse tissues (Kurosaki et al., 1995; Terao et al., 1992). Furthermore, XOR mRNA increases *in vitro* in mouse fibroblasts after treatment with interferon-α and-γ (Falciani et al., 1992), and XOR activity is enhanced in rat pulmonary endothelial cells after culturing with interferon-γ (Dupont et al., 1992). Cytokines such as tumor necrosis factor-α, interleukin-1, and interleukin-6, enhance XOR activity and gene expression in bovine renal epithelial cells (Pfeffer et al., 1994).

Furthermore, it was shown by DNA microarray analysis of gene expression that the XOR gene is upregulated in a rat model of systemic inflammation (Chinnaiyan et al., 2001), thus supporting a role for XOR in the inflammatory process.

4.2. Lactation

The mammary gland represents a good example of tissue- and stage-dependent expression of XOR. Under normal non-lactating conditions, low levels of XOR mRNA, protein and activity are associated with the epithelium in the mouse mammary gland (Kurosaki et al., 1996). During the final phase of pregnancy and the whole period of lactation, there is a striking increase in XOR activity and the corresponding transcript (Kurosaki et al., 1996; McManaman et al., 2000). At the beginning of lactation, XOR is delocalized from the cytoplasm to the apical membranes of the mouse mammary gland epithelial cells, in which the protein is associated with milk fat globule proteins (McManaman et al., 2002). In the only human study using mammary epithelial cells, XOR activity and mRNA expression were increased after stimulation with tumor necrosis factor-α, interleukin-1, and interferon-γ (Page et al., 1998).

Interestingly, mice heterozygous for a loss of function in the XOR gene (*XOR+/-*) are unable to maintain lactation because of membrane defects in the milk fat droplets and disruption of the mammary epithelial cells (Vorbach et al., 2002), indicating that XOR may have a structural role in the development of the mammary gland.

Corticosteroids such as dexamethasone or cortisone transcriptionally induce XOR in mouse mammary gland cells, and the effect is further enhanced by prolactin (Kurosaki et al., 1996; McManaman et al., 2000).

4.3. Oxygen

4.3.1. Tissue oxygen concentration

Hypoxia refers to below-normal levels of oxygen in air, blood, or tissue. Although cells are usually cultured in the laboratory at an ambient oxygen concentration of 21%, cells in the human body are generally exposed to much lower oxygen. Expressed in similar terms, the measured oxygen concentrations *in vivo* would be 4% for the liver (Wolfle et al., 1983), 3% for the renal cortex (Epstein et al., 1994), and as low as 2% for the myocardium (Rumsey et al., 1994). It has been shown that lowering the oxygen concentration of the culture media has beneficial effects on cultured cells, increasing the life-span and division rate of human fibroblasts (Packer and Fuehr, 1977; Storch and Talley, 1988).

4.3.2. Oxygen sensing

Even a small reduction in cellular oxygen concentration results in the activation of

oxygen-responsive genes. It has been suggested that an oxygen sensing mechanism exists in every mammalian cell, but no consensus regarding the nature of this sensor exists.

Hydroxylation of hypoxia-inducible factor-1 (HIF-1) is mediated by a group of HIF-1-specific hydroxylases, and cobalt can inactivate the enzymes by occupying an iron-binding site on the hydroxylases. Because proline hydroxylation requires molecular oxygen, it has been suggested that HIF-1-specific hydroxylases are themselves the "oxygen sensors" (Semenza, 2004).

Other data suggest that the oxygen sensor is a heme protein which undergoes allosteric modification in response to oxygen binding or release (Goldberg et al., 1988). Yet, other studies propose that mitochondria function as oxygen sensors by paradoxically increasing their generation of superoxide during cellular hypoxia (Chandel and Schumacker, 2000).

4.3.3. Transcriptional and post-transcriptional regulation by oxygen

In mammalian cells, HIF-1 has emerged as a key regulator of oxygen homeostasis, playing a central role in responses to hypoxia. HIF-1 is a transcription factor that targets genes involved in such diverse functions as angiogenesis, hormonal regulation, energy metabolism, cellular transport, growth, and apoptosis. The HIF-1 protein consists of α- and β-subunits. The cells maintain relatively stable concentrations of the HIF-1β-subunit, whereas HIF-1α concentrations vary, going up when oxygen levels are low and down when they are normal. It has been proposed that oxygen sensing and signaling occur via a common pathway, leading to the binding of HIF-1 to the consensus HIF-1 DNA binding site present in the hypoxia-responsive elements of oxygen responsive genes, leading to increased transcription (Schofield and Ratcliffe, 2004).

Other than HIF-mediated responses to oxygen, particularly to reactive oxygen species, have been implicated. These include the activation of transcription factors such as nuclear factor-κB and activator protein-1, enhancing gene expression of pro-inflammatory mediators (Rahman and MacNee, 1998). Post-transcriptional responses to oxidative stress are exemplified by heat shock protein Hsp33, which is under oxidative stress control through the formation of disulfide bonds (Graf and Jakob, 2002), and VEGF mRNA, which is stabilized in hypoxia (Claffey et al., 1998).

4.3.4. Regulation of XOR by hypoxia and hyperoxia

Oxygen tension is a significant determinant of XOR expression. In general, hypoxia tends to increase XOR, while hyperoxia decreases the activity of the protein. The regulation of XOR by oxygen has mostly been studied in animal-derived cells.

Hypoxia. Data on the effects of hypoxia on XOR expression are conflicting, showing both transcriptional and post-translational regulation (Table 2). Elevated XOR activity has been documented in a number of studies on anoxic or hypoxic rat, bovine, or mouse endothelial cells (Hassoun et al., 1994; Kayyali et al., 2001; Poss et al., 1996; Terada et al., 1992b), as well as on human umbilical vein endothelial cells (Terada et al., 1992b). XOR activity and mRNA levels are increased in hypoxic rat endothelial cells (Hassoun et al., 1994) and in mouse fibroblasts (Terada et al., 1992b), supporting a role for transcriptional regulation. A putative binding site for HIF-1 has been identified in the 5′-upstream region of the human XOR gene (Hoidal et al., 1997), but evidence of HIF-mediated regulation of XOR has not been published.

In contrast, post-translational regulation of XOR in hypoxia was suggested in bovine endothelial cells, since XOR activity increased, whereas XOR mRNA levels

remained unchanged (Poss et al., 1996). Another potential mechanism for post-translational activation of XOR in hypoxic rat endothelial cells is the phosphorylation of the protein by casein kinase II (involved in cell growth) and p38 (involved in stress activation) (Kayyali et al., 2001).

Hyperoxia. From the literature it seems that high oxygen concentrations regulate XOR at several levels, i.e. by post-translational inactivation of the enzyme or through transcriptional events. For example, in rat endothelial cells, XOR mRNA concentrations were diminished after incubation in hyperoxia, as compared to normoxic cells (Hassoun et al., 1994) (Lanzillo et al., 1996). Conversely, XOR mRNA concentrations were not decreased in hyperoxic mouse fibroblasts, although there was a profound decrease in XOR activity (Terada et al., 1997). Increasing concentrations of reactive oxygen species progressively decrease the activity of purified bovine XOR (Terada et al., 1991), and hyperoxia as well as reactive oxygen species inactivate the enzyme in isolated rat lungs and endothelial cells (Terada et al., 1988).

Table 2. Effect of hypoxia on XOR in cultured cells.

Oxygen (%)	Cell line	XOR			Reference
		Activity	Protein	mRNA	
0	Bovine EC	↑	↔	↔	(Poss et al., 1996)
0	Mouse fibroblasts	↑	↑	↑	(Terada et al., 1997)
	HUVEC	↑	-	-	
	Bovine lung EC	↑	-	-	
	Human intestinal	↑	-	-	
1	Bovine lung EC	-	-	↑	(Partridge et al., 1992)
3	HUVEC	↔	-	-	(Hassoun et al., 1994)
	Bovine BASMC	↑	-	-	
	Rat lung EC	↑	-	-	
	Rat fat pad EC	↑	-	↑	
3	Rat lung EC	-	-	↑	(Lanzillo et al., 1996)
3	Rat lung EC	↑	-	-	(Kayyali et al., 2001)
0-10	Bovine lung EC	↑	-	-	(Terada et al., 1992b)

-, not analyzed; ↔, no change; ↑, increased; ↓, decreased; EC, endothelial cells, HUVEC, human umbilical vein endothelial cells; BASMC, bovine aortic smooth muscle cells.

5. Role of XOR in human pathophysiology

5.1. Genetic deficiency of XOR

Inherited XOR deficiency is classified into: 1. classical xanthinuria type I, lacking only XOR; 2. classical xanthinuria type II, lacking XOR and AO activity; 3. molybdenum cofactor deficiency, lacking XOR, AO, and sulfite oxidase. Most affected individuals with xanthinuria type I and II have no symptoms, but some patients may develop urinary tract calculi, acute renal failure, or myositis due to accumulation of insoluble xanthine (Raivio et al., 2001). Type I xanthinuria is a rare autosomal recessive disorder and a single-nucleotide mutation has been shown to be responsible for the disease (Ichida et al., 1997). A molybdenum cofactor sulfurase is responsible for inserting the essential sulfur atom into the molybdenum cofactor of XOR and AO, and a mutation of this sulfurase is apparently responsible for at least some cases of type II xanthinuria (Ichida et al., 2001). Recently, the generation of XOR knockout mice was reported, but a homozygous deletion of the *XOR* gene turned out to be lethal in the embryonic stage (Vorbach et al., 2002). This is in apparent contrast to human xanthinuria, since these patients do not seem to have a shortened life-span. In contrast to xanthinuria type I and II, molybdenum cofactor deficiency is associated with severe neurological disorders, apparently due to concomitant lack of sulfite oxidase (Raivio et al., 2001).

5.2. Ischemia-reperfusion

XOR has been linked to the pathophysiology of reoxygenation after hypoxia, i.e. ischemia-reperfusion (I/R) injury (McCord, 1985). Examples of such clinical situations include myocardial infarction, stroke, and organ transplantation. According to the hypothesis, a large amount of hypoxanthine is released during ischemia due to the depletion of ATP (Saugstad, 1975). Concurrently, XOR is converted from the XDH to the oxygen radical producing XO form. Upon reperfusion, when oxygen is reintroduced, XO oxidizes the accumulated hypoxanthine to produce toxic oxygen radicals that cause tissue damage (McCord, 1985). Initial evidence supporting this role for XOR in I/R injury was primarily indirect, based on the observation that allopurinol or oxypurinol, inhibitors of XOR, diminished cellular damage after reperfusion in the intestine (Granger et al., 1981; Parks et al., 1982). The time course for the dehydrogenase to oxidase conversion during ischemia varies depending on the animal model, organ, and cell type. For example, the conversion is rapid in isolated rat Kupffer cells and slow in aortic endothelial cells (Wiezorek et al., 1994).

The main problem is whether XOR actually exists in the human organs that may suffer reperfusion injury, particularly the heart and brain, and whether the dehydrogense (XDH) form of the enzyme in fact is converted to the reactive oxygen species forming oxidase (XO) under these circumstances. Thus, although the proposed role for XOR in the pathogenesis I/R injury is attractive, it seems that conclusive evidence to support the hypothesis has not been presented.

5.3. Cancer

5.3.1. XOR and cancer

Although XOR activity has been determined in a number of animal tumors and in a few human tumors (Table 3), little is known about the expression of XOR in cancer. XOR activity is significantly reduced in rat hepatomas (Ikegami et al., 1986), including the slowest growing and well differentiated tumors (Prajda and Weber, 1975). Mouse mammary tumors (Lewin et al., 1957) and colon carcinomas (Weber et al., 1978) show considerably decreased XOR activities compared to analogous normal tissue. In addition, XOR activity is decreased in human hepatocellular (Stirpe et al., 2002) and renal cell carcinoma (Durak et al., 1997), as compared to corresponding normal tissue. In a rat model for chemically induced mammary

gland carcinoma, using large-scale gene expression profiling by cDNA microarrays, the XOR gene was down-regulated compared to the normal and lactating gland (Shan et al., 2002).

The expression of XOR in human cancer has previously been examined only in one study involving breast cancer (Cook et al., 1997). This study included samples of normal breast (n = 6), ductal carcinoma *in situ* (n = 5), and infiltrating ductal carcinoma (n = 4). The results showed that no immunohistochemically detectable XOR was observed in any of the intraductal *in situ* carcinomas or invasive breast carcinomas studied.

Taken together, XOR is downregulated in cancerous tissue in virtually all rodent and human tumors studied. Interestingly, enzymes involved in the purine synthetic pathway were upregulated in tumors which showed decreased XOR activity as compared to corresponding normal tissue (Prajda et al., 1976; Prajda and Weber, 1975), indicating an altered balance between purine catabolic and anabolic pathways.

Table 3. XOR activity, protein and gene expression in rodent (rat or mouse) and human tumors.

Origin	XOR			Reference
	Activity	Protein	Gene	
Rodents				
Mammary ca	↓	-	-	(Lewin et al., 1957)
Mammary ca	-	-	↓	(Shan et al., 2002)
Hepatoma	↓	-	-	(Prajda and Weber, 1975)
Hepatoma	↓	-	-	(Ikegami et al., 1986)
Colon ca	↓	-	-	(Weber et al., 1978)
Renal ca	↓	-	-	(Prajda et al., 1981)
Humans				
Mammary ca	-	↓	-	(Cook et al., 1997)
Hepatoma	↓	-	-	(Stirpe et al., 2002)
Renal ca	↓	-	-	(Prajda et al., 1981)
Renal ca	↓	-	-	(Durak et al., 1997)
Prostate ca	↔	-	-	(Biri et al., 1999)
Head and neck ca	-	-	↑	(Belbin et al., 2002)

-, not analyzed; ↔, no change; ↑, increased; ↓, decreased; ca, carcinoma

5.3.2. Prognostic and predictive markers for breast cancer

Breast cancer is the most common malignant disease in women (Parkin et al., 1999). In 2002, the age-adjusted incidence rate of breast cancer in Finland was 85 cases per 100.000 females and a total of 3760 women were diagnosed with the disease (Finnish Cancer Registry, 2004). Breast cancer is a heterogenous disease, with marked variations in malignant potential and metastatic capacity (Donegan and Spratt, 2002). The risk of dying from breast cancer varies considerably between patients, from almost no risk for patients with *in situ* cancer (Warnberg et al., 1999) to 70% or more risk for relapse within five years from diagnosis in patients with poorly differentiated tumors, e.g. >10 positive lymph nodes, and tumor size >5 cm (National Cancer Institute, released April 2003, based on the November 2002 submission).

Axillary lymph node status and tumor size are the most powerful predictors of disease-free survival in breast cancer (Goldhirsch et al., 2003), and histopathological grade adds independent prognostic information (Elston and Ellis, 1991). Estrogen and progesterone receptor status of the breast tumors are established biomarkers, mainly used as predictive factors for response to therapeutic and adjuvant hormone treatment (Fitzgibbons et al., 2000). Determination of human epidermal growth factor-2 (Her-2, *ERBB2*) overexpression or gene amplification is recommended to identify patients who may benefit from therapy with monoclonal antibodies (Trastuzumab) against the receptor (Bast et al., 2001). Although alterations in the tumor suppressor gene *P53*, and proliferation associated Ki-67 protein expression have been associated with poor prognosis in breast cancer, there is no consensus that testing should be performed in routine practice (Fitzgibbons et al., 2000). Numerous other biomarkers have been evaluated as potential prognostic factors in breast cancer (Hayes et al., 1996), and novel approaches to estimation of prognosis and prediction of therapeutic response include large-scale gene expression profiling (Sorlie et al., 2003; van 't Veer et al., 2002)

AIMS OF THE STUDY

We hypothesized that because of its unique metabolic role and the structural characteristics of the promoter of its gene, the regulation of the expression of human XOR and its pathophysiological role are specific to humans and cannot be extrapolated from animal experiments. We were also interested in the relationship of XOR expression to breast cancer, given the inducibility of the enzyme during lactation and its low or absent expression in several tumors. Therefore, the aims of the study were:

1. to characterize the molecular forms and activity to protein relationships of XOR in human liver and intestine,

2. to document the cellular localization of XOR in normal human tissues,

3. to evaluate the effect of oxygen on XOR activity, protein, mRNA, and promoter activity in cultured human epithelial cells,

4. to investigate the expression of XOR in malignant breast epithelial cells in a large cohort of human breast cancer, and to assess its potential prognostic value.

MATERIALS AND METHODS

1. Patients and samples

1.1. Human tissue specimens and serum samples (I, II)

Samples of fetal liver and intestine were obtained from elective terminations (10-21 weeks gestation), and neonatal samples (25-35 weeks gestation) were from autopsies performed within two hours after death. The study protocol was approved by the Ethical Committees of the Hospital for Children and Adolescents, and the Department of Obstetrics and Gynecology, University of Helsinki, Helsinki, Finland.

Adult liver tissue was obtained from the extra tissue at partial liver transplantation, and serum samples were from healthy adults and newborn infants (I).

For the localization of XOR in healthy human tissues the samples were from: 1. the healthy margins of surgical specimens from liver, breast, kidney, and cerebral cortex; 2. transcutaneus biopsies of liver and breast; 3. hearts explanted from patients with hyperthrophic cardiomyopathy undergoing cardiac transplantation (II). The criterion for inclusion in the study was that the tissue was found to be normal upon histologic examination by a trained pathologist.

1.2. Patients, exclusion criteria, and follow-up (IV)

To study the expression of XOR in breast cancer, five well-defined geographical regions comprising about 50% of the Finnish population were selected. Women diagnosed with breast cancer within these regions in 1991 or 1992 were identified from the files of the Finnish Cancer Registry. A computer search recognized 2930 women, which constituted 53% of all breast cancers diagnosed in Finland within this time period.

For inclusion in the study the following minimum information was required to be available: the date of diagnosis, age at diagnosis, history of other malignancies, postsurgical primary tumor size, axillary nodal status, follow-up data, and the vital status data at the end of follow-up.

The total number of patients entered into the database was 2842. The following patients were excluded from the study: patients with lobular (n = 17) or ductal (n = 186) carcinoma *in situ*, those with distant metastases at diagnosis (n = 136), women with synchronous or metachronous bilateral breast cancer (n = 261) or history of other malignancy (except for basal cell carcinoma or cervical carcinoma in situ, n = 235), and women who did not undergo breast surgery (n = 42), leaving 1983 women with unilateral invasive breast carcinoma for the study.

The median follow-up of patients alive at the end of follow-up is 9.5 years.

1.3. Cell lines and culture (III)

Transformed human bronchial epithelial cells (BEAS-2B) and human embryonic kidney cells (293T) were obtained from the American Type Culture Collection (Manassas, VA, USA) and Dr. Kalle Saksela (University of Tampere, Finland) respectively. BEAS-2B is a stable, SV-40 transformed normal human bronchial epithelial cell line, which retains the ability to undergo squamous differentiation (Ke et al., 1988; Reddel et al., 1988). BEAS-2B cells were cultured in serum-free hormone-supplemented bronchial epithelial cell growth medium (Cytotech ApS, Hellebaek, Denmark) at 37 °C in a 5% CO_2 humidified

atmosphere. 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco, Europe, Paisley, UK).

For exposure of confluent cells to hypoxia (0.5 or 3% O_2) or hyperoxia (95% O_2), a preanalyzed gas mixture (5% CO_2, specified O_2 %, balance N_2, AGA, Finland) was infused into airtight humidified chambers (Billups-Rothenburg, Del Mar, CA) in which the cells were grown.

2. Methods

2.1. Sample preparation

2.1.1. Tissue samples (I, II, IV)

Human tissue specimens were snap frozen in liquid nitrogen and stored at -70°C. Thawed samples were homogenized in 100 mM Tris-HCl (pH 8.0) containing mercaptoethanol, and sonicated on ice. The sonicates were then centrifuged and the resulting supernatants were used for further analysis (I).

For immunohistochemistry, fresh tissue specimens were immediately immersed in 10% neutral buffered formalin and embedded in paraffin according to standard histopathological procedures (II, IV).

2.1.2. Cultured cells (III)

For enzyme activity measurements and Western blotting, cells were washed twice with PBS, mechanically harvested, and centrifuged. The cell pellets was resuspended in 50 mM potassium phosphate buffer, pH 7.8, containing 0.5 mM dithiothreitol and 1 mM EDTA, sonicated on ice, and centrifuged.

For mRNA analysis, total RNA was extracted from BEAS-2B cells using the acid phenol-chlorophorm method (Chomczynski and Sacchi, 1987).

2.2. Enzyme activity measurements (I, III)

2.2.1. XOR activity (I, III)

After electrophoresis of purified XOR preparations in 7.5% agarose gel under non-denaturing conditions, XO activity was visualized by incubating the gel in 1.35 mM xanthine and 0.02% nitroblue tetrazolium diluted in 0.05 M phosphate buffer for 30 min (I).

The combined XDH and XO activities (I) from tissues were measured using ^{14}C-hypoxanthine as substrate, and the formation of ^{14}C-xanthine and ^{14}C-uric acid was followed by thin-layer chromatography. The spots containing hypoxanthine and xanthine plus uric acid were scraped off and counted using liquid scintillation (Vettenranta and Raivio, 1990).

For XOR activity measurements (III), cell supernatants were incubated for 60 min in the presence of ^{14}C-xanthine (0.1 mM, specific activity 58 mCi/mmol; NEN, Life Science Products Incorporation, Boston, MA). The combined XDH and XO activities were measured in the presence NAD^+ (400 μM) and for XO activity NAD^+ was omitted. The uric acid produced was separated by HPLC (Shimadzu, Kyoto, Japan) with a reverse-phase column and eluted with 50 mM potassium phosphate, pH 4.5. Finally, radioactivity was quantified with a Radiomatic Flow Scintillation Analyzer (Packard Instrument, Meriden, CT, USA). XOR activity was expressed as nmol/min per mg total protein, which was analyzed using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

Inactivation of XOR by hydrogen peroxide was studied by using purified bovine XOR (Biozyme Laboratories, South Wales,UK). XOR activity was determined spectrophotometrically at 295 nm, by following the production of uric acid over 3 min before and after 30 min of incubation with H_2O_2 (III).

2.2.2. 2, 6-dichlorophenolindophenol reduction and NADH oxidation (III)

Transfer of electrons from xanthine to the artificial electron acceptor 2, 6-dichlorophenolindophenol (DCPIP) was measured spectrophotometrically by monitoring the absorbance of DCPIP at 600 nm. The oxidation of NADH was monitored spectrophotometrically at 340 nm.

2.3. Purification of XOR and production of anti-XOR antibodies (I)

Buffered human breast milk was heated to 50 °C with Triton X-100, fractionated with ammonium sulfate between 20 and 70 g/l, the resulting pellet was resuspended in 5 mM phosphate, and dialyzed against the same buffer. The proteins were separated in a Diethylaminoethyl (DEAE)-Sepharose column and the enzyme was eluted with a linear gradient of 5-40 mM phosphate. The fractions containing the highest XOR activities were pooled, after which 20% hydroxyapatite was added, and the gel was eluted stepwise with phosphate. The eluates with the highest specific XOR activities were pooled and subjected to HPLC using a hydroxyapatite column. The purification was monitored by calculating absorbances at 280 and 450 nm, by SDS-PAGE, and by measuring XOR activities.

For production of XOR antiserum, three rabbits were immunized three times at 3-week intervals with purified human milk XOR (50 µg). The rabbits were bled two weeks after the final immunization, and the antiserum was purified by solid phase absorption with the globulin fraction of human serum and with major milk proteins (β-caseins, β-lactoglobulin, and lactoferrin).

2.4. ELISA (I, III)

For ELISA measurements, microtiter plates were coated with the purified XOR antiserum over night and residual binding sites were blocked with 2% BSA-TBS. Plates coated with 2% BSA-TTBS were used as controls for non-specific binding. XOR standards (1-50 ng/mg) and samples (diluted 1:5 or more) were diluted in 1% BSA-TTBS, and incubated over night at room temperature. After washing with TTBS, alkaline phosphatase conjugated anti-XOR antibodies (diluted 1:300) was added into the wells and incubated for 4 h. The substrate (paranitrophenylphosphate and diethanolamine) was added, and the plates were incubated for 45 min at 37°C. The absorbances were calculated at 405 nm by a Multiscan MS (Labsystems, Helsinki, Finland).

2.5. Electrophoresis and Western blotting analysis (I, III)

For Western analysis, the proteins were boiled in a buffer containing β-mercaptoethanol (5 min at 95°C), and loaded onto a 7.5% SDS-polyacrylamide gel. After electrophoresis the proteins were transferred onto Immobilon-P membranes (Millipore, Bedford, MA). Nonspecific binding was blocked with bovine skimmed milk 2-5% (w/v) for 60 min, after which the membranes were incubated with the XOR antiserum diluted 1:200 (I) or 1:300 (III) for 2 h. The membranes were then incubated with alkaline phosphatase–conjugated anti-rabbit antibody (diluted 1:100) (I) or with horseradish peroxidase-conjugated anti-rabbit antibody (diluted 1:5000, Jackson-Immuno Research Laboratories, West Grove, PA, USA) (III). For visualization, the membranes were incubated with the alkaline phosphatase substrate 5-bromo, 3-inolylphosphate (BCIP)/nitrobluetetrazolium (NBT) (I) or by using enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK) (III).

2.6. Immunohistochemistry (II, IV)

The sections (4 or 5µm) were deparaffinized and rehydrated, and endogenous peroxidase was quenched by incubation of slides in 0.3

(II) or 1% (IV) (v/w) hydrogen peroxide for 30 or 10 min respectively.

For localization of XOR in normal tissues (II), the sections were heated in 10 mM citric acid (pH 6.0) in a microwave oven for 10 min to enhance antigen, and incubated with 20% normal goat serum (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) to block nonspecific binding. The sections were then incubated with anti-XOR antibodies diluted 1:200 in PBS overnight at +4°C. Biotinylated goat anti-rabbit antibody (Vectastain Elite ABC kit) was added onto the sections for 1 h and then incubated with an avidin: biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit). For visualization, 0.05% 3,3´-diaminobenzidine (DAB) (Sigma Chemical Co., St. Louis, MO) was added onto sections.

For detection of the XOR in normal and tumor tissue (IV), the slides were heated in Target Retrieval Solution, pH 6.0 (DAKO, Carpinteria, CA) at 95-97°C for 30 min. The sections were incubated with anti-XOR antibodies diluted 1:50 in Blocking Solution (Powervision, Immunovision, Inc., Daly City, CA) overnight at +4ºC. For detection, an antimouse-peroxidase polymer (dextran) and diaminobenzidine as a chromogen (Powervision) was added onto the sections. Finally, the slides were counterstained with hematoxylin diluted 1:10 and embedded.

To exclude the possibility of non-specific reactions of secondary antibodies, control sections were processed without primary antiserum (II, IV). In addition, sections were stained with the preimmune serum from the immunized rabbit (II, IV), and also with the XOR antiserum after absorption with purified XOR (II).

2.7. Preparation of tumor tissue array blocks and scoring of XOR staining (IV)

Representative tumor regions in formalin-fixed paraffin-embedded samples were first defined in hematoxylin-eosin-stained sections and marked. Tumor tissue array blocks were then produced by punching a 0.6 mm tissue cylinder through a histologically representative area of each "donor" tumor block, which was then inserted into an empty "recipient" tissue array paraffin block using a specific instrument (Kononen et al., 1998). From the tumor samples available 19 tissue array blocks were prepared, each containing 50 to 144 tumor samples.

Evaluation of the tissue array slides was aided by the use of a computer-controlled and motorized specimen stage (EcoDrive, Märzhauser Inc., Germany) installed on a light microscope (Olympus BX50). XOR staining of the cytoplasm and nucleus were scored separately. Cytoplasmic XOR expression was scored as follows: strong staining intensity comparable to that of normal breast epithelial cells; clearly decreased staining intensity as compared to that of the normal breast epithelium; negative, no visible staining for the XOR in more than 90% of the cancer cells.

Nuclear XOR staining intensity was compared to that of nuclei in normal breast cells and scored as follows: strong staining; moderate staining; and no visible staining. Strong and moderate staining of the nuclei represents increased XOR expression as compared to the nuclei of the normal breast epithelial cells.

2.8. Ribonuclease protection assay (III)

Ribonuclease protection assay (RPA) was performed using a commercially available kit according to the manufacturer's recommendations (RPA II kit; Ambion, Austin, TX, USA), using 20 μg of total cellular RNA. The XOR antisense riboprobe, corresponding to the nucleotides 405-789 of the human XOR cDNA, was P^{32}-labeled. To normalize for RNA content, the samples were hybridized with RNA probes transcribed from human β-actin cDNA.

2.9. Assessment of cell injury (III)

The cell suspension was incubated in 0.4% trypan blue for 5 min, and counted in a Bürker cell counting chamber. Trypan blue-negative cells were considered viable.

2.10. Promoter constructs and reporter gene analysis (III)

For studying the response of the XOR promoter to hypoxia, 293T cells were transfected with human XOR promoter fragments and with a hypoxia-responsive reporter gene construct (HRE-luc) carrying three tandem copies of the erythropoietin hypoxia-responsive element coupled to luciferase (kindly provided by Dr. Pekka Kallio) as previously described (Martelin et al., 2000). The transfections were carried out using the FuGene6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN), and transfection efficiency was controlled for by cotransfection with a β-galactosidase-carrying construct (pCMVβ, Clontech, Palo Alto, CA). After transfection the cells were exposed to either 0.5 or 21% oxygen for 24 h. Luciferase and β-galactosidase were then measured (Martelin et al., 2000).

2.11. Statistics (I, III, IV)

Pearson´s product-moment correlation was used to estimate the relationship between two continuous variables identified as XOR activity and protein concentration (I).

Enzyme activities before (control) and after exposures were compared using the unpaired Student´s t-test. The values are shown as means ± SD (III).

Associations between XOR expression and clinicopathological parameters were analyzed using the chi-square test, and life-tables were calculated according to the Kaplan-Meier method. Distant disease-free survival (DDFS) was calculated from the date of the diagnosis to the first detection of metastases outside the locoregional area, or death from breast cancer. The logrank test was used to calculate the statistical significance of the difference in recurrence-free survival between two groups, and the logrank test for trend in case of three or more categories. Multivariate survival analysis was calculated using the Cox proportional hazards model (IV). P-values were two-tailed and values <0.05 were considered significant.

RESULTS

1. Organ distribution and molecular forms of human XOR (I)

1.1. The purified XOR preparation and anti-XOR antibodies

To produce polyclonal anti-XOR antibodies, we purified XOR from human milk, and the protein was eluted from a DEAE-Sepharose column as a single peak.

The purified XOR preparation was analyzed with electrophoresis under non-denaturing conditions, and one band with a molecular size of 300 kDa was observed. The XOR activity band corresponded to this band, which was partially sequenced and the amino acid sequence corresponded to the human XOR sequence deduced from the known cDNA structure. When subjected to an SDS-PAGE analysis the purified XOR preparation showed a main band of 143 kDa, with additional bands of 125, 87, and 59 kDa (I, Figure 2).

After absorption of the anti-XOR antiserum, produced in rabbits, with the globulin fraction of human serum and lactoferrin, the antiserum gave one major band in Western analysis with the purified XDH/XO preparation and human milk, with an estimated molecular weight of 143 kDa and additional minor bands of 125, 87 and 59 kDa (I, Figure 3). The bands corresponded well with the protein bands in SDS-PAGE (I, Figure 2). Lactoferrin, α- and β-caseins, β-lactoglobulin, and normal human serum (I, Figure 3) were negative upon Western blotting.

1.2. XOR protein in tissue homogenates

The anti-XOR antibodies recognized bands of similar size in Western blots of the purified preparation and human milk (I, Figure 3). In fresh liver homogenates treated with antiproteases, the three largest bands were observed, in the intestine only the two largest (I, Figure 4). Brain, heart, skeletal muscle, and serum did not show immunoreactivity, whereas lung and kidney showed one faint band of 143 kDa in some samples (I, Figure 4) After trypsin treatment of liver homogenate and the purified XOR preparation, the main 143 and 125 kDa bands disappeared and the 87 kDa band became more intense (I, Figure 6). The same pattern was seen after incubation of liver homogenate with protease inhibitors at 37°C for 20 min.

1.3. XOR activity to XOR protein relationship

By ELISA, XOR concentrations were 146 ± 70 ng/mg total protein (mean ± SD) for the liver and 556 ± 320 ng/mg total protein for the intestine. Lung and kidney had clearly smaller although measurable amounts of XOR (40-80 ng/mg total protein), whereas the XOR protein concentrations in brain, heart, skeletal muscle, and serum were below the detection limit. When XOR activities for the intestine and liver were compared with XOR protein concentrations in each of the samples, a linear correlation was found in both of the organs, $r = 0.76$ for the liver and $r = 0.91$ for the intestine (I, Figure 5). The specific activities were approximately 3 μmol/min/mg XOR protein for both organs, whereas the specific activity for human milk was only 20% of that in the liver and intestine.

2. Localization of human XOR in normal human tissues (II)

Using polyclonal anti-XOR antibodies raised against human milk XOR (I), the protein was localized in normal human tissues (Table 4). Strong staining of XOR was seen in the epithelial cells of the lactating mammary

gland and the proximal intestine (II, Figure 1). Also the periportal hepatocytes as well as the Kupffer cells of the liver showed intense staining. Moderate XOR expression was observed in the epithelial cells of the non-lactating mammary gland and in the pericentral hepatocytes, whereas pericentral Kupffer cells did not show XOR immunoreactivity (Figure 4). The cytoplasm of the capillary endothelial cells in the non-lactating and lactating mammary gland, proximal intestine, skeletal muscle, and kidney showed moderate XOR expression. In addition, the arterioles of the mammary gland showed XOR immunoreactivity (II, Figure 2). All cells in the heart, brain, and lungs were below the detection limit (II, Figure 1).

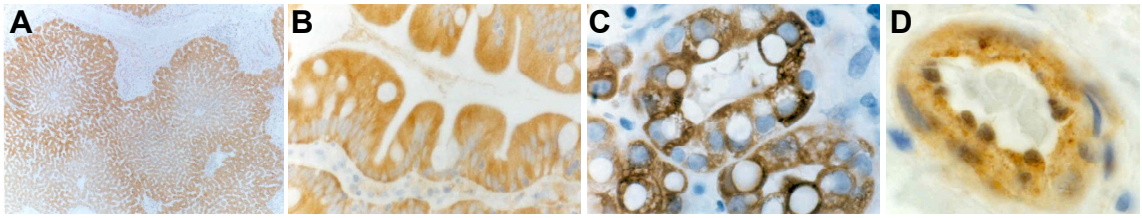

Figure 4. Immunohistochemical localization of XOR in normal human liver (A), proximal intestine (B), and lactating breast (C). An arteriole from lactating breast is positively stained (D).

Table 4. Localization and staining intensity of XOR in normal human tissues (II, IV).

Tissue	XOR Localization
Non-lactating mammary gland	Acinar cells of terminal ducts (+) and large ducts (+), capillary endothelial cells (+) and arterioles (+), myoepithelial cells (-)
Lactating mammary gland	Epithelial cells of all ducts (++), endothelial cells of capillaries (+) and arterioles (+), myoepithelial cells (-)
Liver	Periportal hepatocytes and Kupffer cells (++), pericentral hepatocytes (+), portal vein endothelium (-), hepatic artery endothelium (-)
Proximal intestine	Enterocytes and goblet cells (++), capillary endothelial cells (+)
Skeletal muscle	Muscle cells (-), endothelial cells of capillaries and venules (+)
Kidney	Capillary endothelial cells in connective tissue (+), tubules and glomeruli (-)
Heart	(-)
Brain	(-)
Lung	(-)

Strong (++), moderate (+), and no (-) staining of XOR.

3. Regulation of XOR by oxygen (III)

3.1. Post-translational regulation of XOR in hypoxia and hyperoxia

XOR activity in BEAS-2B cells grown in hypoxia (0.5 and 3% O_2) was three- to eightfold higher respectively, compared to cells grown in normoxia (21% O_2) (III, Figure 1), whereas XOR concentrations and mRNA levels were unaltered (III, Figure 3). Incubation of cells with cobalt chloride did not alter XOR activity. The XOR promoter activity remained unchanged in hypoxic as compared to normoxic cells (III, Figure 2).

Hyperoxia (95% O_2) abolished cellular XOR activity, whereas XOR concentrations did not change as compared to normoxia (III, Figure 5). Reoxygenation of cells after incubation in hypoxia returned XOR activity to basal levels (III, Figure 4).

The oxidase form of the enzyme, XO, represented ~ 20% of the combined XDH and XO activity at all oxygen concentrations.

3.2. Inactivation of XOR active center by high oxygen

To study the mechanism of inactivation of XOR in hyperoxia, the function of the main redox centers of XOR was evaluated. The function of the FAD center was apparently unaffected since the oxidation of NADH, which is dependent on the FAD center, was similar in sonicates of cells grown in normoxia, hypoxia, and hyperoxia. Incubation of purified XOR with hydrogen peroxide decreased XOR and DCPIP activity in parallel, suggesting that the molybdenum center of XOR lost its capacity to transfer electrons (III, Figure 6).

Table 5. Summary of the effects of hypoxia (0.5-3% O_2), hyperoxia (95% O_2), and cobalt chloride on XOR in BEAS-2B and 293T cells (promoter experiments).

Agent	XOR			
	Activity	Protein	mRNA	Promoter
Hypoxia	↑	↔	↔	↔
Hyperoxia	↓	↔	-	-
Cobalt chloride	↔	-	-	-

-, not analyzed; ↔, no change; ↑, increased; ↓, decreased

4. XOR expression and its clinical correlates in breast cancer (IV)

4.1. XOR expression in breast cancer

Forty-three percent of the 1262 tumors (n = 543) studied showed strong XOR staining in the cytoplasm, similar to the staining in normal breast epithelial cells, and 50% (n = 631) showed decreased XOR staining as compared to normal breast epithelium. Seven percent (n = 88) of the tumors showed minimal or no XOR immunostaining in the cytoplasm and were scored negative (Table 6 and Figure 5).

In 21% of the tumors XOR was strongly expressed in the nuclei, whereas 30% showed moderate, and 49% no nuclear XOR staining comparable to the nuclei of normal breast epithelial cells.

To evaluate the heterogeneity of XOR expression, 20 sections of whole tumors were stained with the anti-XOR antibodies. Heterogeneity was minimal indicating that the staining results obtained from the tissue microarray cores are representative for the entire tumor.

Table 6. Summary of XOR expression in the 1262 breast tumors studied.

Localization	XOR Expression (%)		
	Strong	Moderate	Negative
Cytoplasm	43	50	7
Nucleus	21	30	49

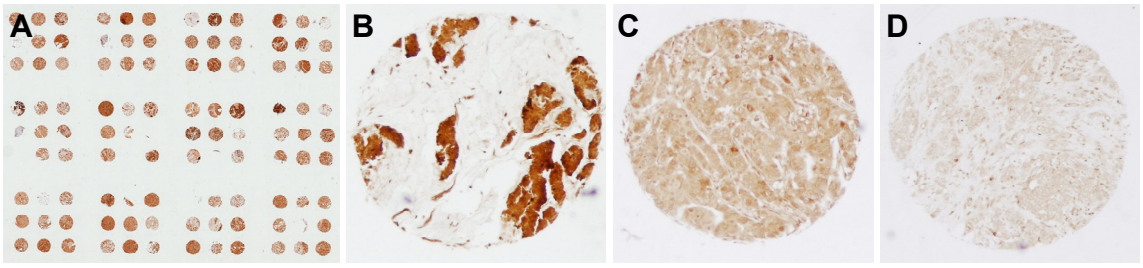

Figure 5. Immunohistochemical staining of XOR in breast cancer specimens on tissue microarray (A) as described in "Materials and Methods". Examples of strong (B), moderate (C), and no (negative) (D) immunoreactivity in the cytoplasm.

4.2. Association of decreased XOR expression with clinicopathological characteristics and outcome

Decreased cytoplasmic XOR expression, as compared to the normal breast epithelium, was associated with clinicopathological variables such as large tumor size (P = 0.043), large number of positive axillary lymph nodes (P = 0.022), and poor histologic grade of differentiation (P = 0.0002), but not with estrogen or progesterone receptor status, Ki-67 antigen, p53 protein, or *ERBB2* amplification (IV, Table 1).

Patients with no cytoplasmic XOR expression had an unfavorable distant disease-free survival (DDFS) compared to those with strong or moderate XOR expression (P <0.0001; RR = 2.2) (Figure 6 and IV, Table 2).

In subgroup analysis, absence of cytoplasmic XOR expression predicted poorer survival in patients with node-negative disease, in patients with small tumor size, in patients with tumors of ductal type, and in hormone receptor positive and negative disease (IV, Table 2 and Figure 4).

In a Cox multivariate analysis, absence of cytoplasmic XOR expression (P = 0.027; RR = 1.69), number of positive lymph nodes, tumor size, histological grade, and progesterone receptor status associated independently with prognosis (IV, Table 3A). In a subgroup of patients with node-negative breast cancer, absence of XOR also emerged as an independent prognostic factor (P = 0.003; RR = 2.79) in addition to tumor size and histological grade (IV, Table 3B), whereas XOR expression did not retain independent prognostic significance among patients with node-positive disease (IV, Table 3C).

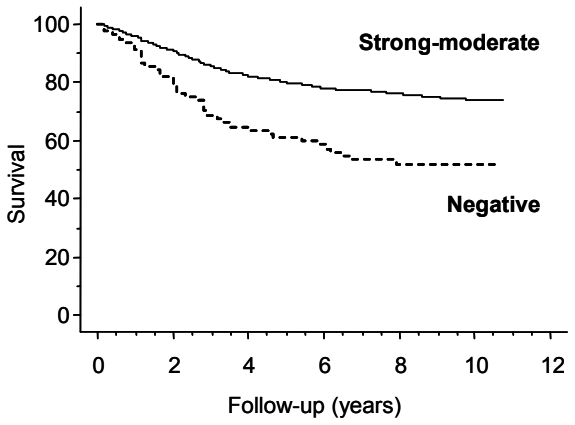

Figure 6. Distant disease-free survival of 1262 patients with breast cancer according to XOR expression. —, moderate and strong (n = 1169); ----, negative (n = 93). P < 0.0001 (logrank test for trend).

DISCUSSION

1. Molecular forms of human XOR (I)

1.1. XOR protein in tissue homogenates and serum

In a study on the proteolysis of rat liver XOR by trypsin, the protein was partially cleaved into fragments of 85, 40, and 20 kDa, which did not dissociate from each other under nonreducing conditions (Amaya et al., 1990). Similar fragments or combinations of these could produce the pattern of bands we observed under reducing conditions. The differences in the number of XOR bands and sizes observed by us and others may also be due to artifacts in sample preparation or purification of the protein. For example, it was shown that after freezing and thawing of human liver bands of 150, 130, and 85 kDa appear in Western blots, whereas fresh liver XOR remains intact for up to 24 h with no signs of proteolysis (Saksela et al., 1999). Although we used fresh liver, and the samples were stored at -70°C in the presence of inhibitors of proteolysis, the homogenates showed multiple bands on Western blotting. It cannot be ruled out that the minor bands are artifacts due to freezing and thawing.

To determine whether inactive XOR is present in human liver and intestine, XOR concentrations and activities were analyzed. The good correlation between XOR activity and XOR protein indicates that the relative amounts of inactive enzyme forms must be small and/or in constant proportion under varying conditions. The delay between death and sample processing may have affected the activity or immunoreactivity of XOR. However, since XOR activity and XOR protein correlated so well in the liver and intestine and during different developmental stages, it is unlikely that postmortem degradation affected the XOR antigen.

In line with previous data showing undetectable XOR activity in normal human serum (Al-Khalidi and Chaglassian, 1965), we could not detect XOR protein in serum samples by Western blotting. We were thus unable to confirm a previous study reporting significant baseline concentrations of XOR in normal human serum (Adachi et al., 1993).

1.2. Specificity of anti-XOR antiserum

The anti-XOR antibodies revealed one major and several smaller bands upon Western blotting of purified XOR and liver homogenate. However, several considerations indicate that the protein used for immunization is, for all practical purposes, pure and that the antiserum produced is specific. XOR purified from human milk and used for immunization revealed only one major band which showed XOR activity after electrophoresis under non-denaturing conditions. The original antibody reacted with lactoferrin, but this reactivity was removed by absorption with lactoferrin. Major milk proteins such as β-caseins and β-lactoglobulin did not react with the anti-XOR antibodies upon Western blotting.

Moreover, when the main protein band of the purified milk preparation was eluted from the non-denaturing gel and partially sequenced, the sequence was identical with that reported for the rat XOR amino acid sequence (Amaya et al., 1990) but showed one amino acid difference from the human sequence (Ichida et al., 1993). Human serum did not show immunoreactivity on Western blotting or ELISA, showing that the anti-XOR antiserum does not show cross-reactivity with serum proteins. Finally, the fact that XOR activity and XOR protein in homogenates of liver and intestine correlate

so well, also suggests that the antiserum is specific for the protein.

2. Localization of the XOR in normal human tissues (II)

The results describing XOR localization in normal human tissues are essentially in accord with previous data, showing high XOR activities in the human liver, intestine, and mammary gland and no or minimal activity in other organs. What appears to be clear is that the enzyme is strictly regulated, its expression being limited to the epithelial cells of the intestine and mammary gland, hepatocytes, and capillary endothelial cells of some organs.

2.1. Liver, intestine, and mammary gland

We showed that, in the liver, XOR is strongly expressed in the periportal hepatocytes, whereas pericentral hepatocytes show clearly lower XOR expression. It is known that periportal and pericentral hepatocytes differ in the expression profile of many proteins (Jungermann, 1982). Hepatocytes receive diverse signals as a result of the gradients established due to passage of oxygen, substrates, and hormones through the liver, which may explain the patchy appearance of XOR in this organ. XOR activity has been localized to pericentral as well as periportal hepatocytes in postmortem human liver (Kooij et al., 1992), whereas in another study zonation of the XOR distribution in the liver was absent (Moriwaki et al., 1993).

In our study, XOR was strongly expressed in Kupffer cells of three of the nine liver samples examined. This could be a consequence of an upregulation of XOR by inflammatory mediators, which have the capacity to induce the protein at a transcriptional level (Kurosaki et al., 1995; Terao et al., 1992). Alternatively, XOR may have been taken up from the portal circulation by Kupffer cells, which are well known to be phagocytic.

The strong XOR expression in goblet cells and enterocytes of the intestine in our study is in line with a previous report (Kooij et al., 1992). The expression of XOR mRNA in the mouse duodenal epithelium (Kurosaki et al., 1995) further supports our findings. However, in bovine intestinal epithelium XOR protein was not detected, whereas, in agreement with our results, capillary endothelial cells in the same organ were shown to express XOR (Jarasch et al., 1981).

Our results showing intense XOR expression in the mammary epithelium during lactation is in accordance with studies showing strong XOR expression in bovine (Jarasch et al., 1981) and mouse (McManaman et al., 2000; McManaman et al., 2002; Vorbach et al., 2002) lactating mammary epithelium. Also in line with our study, increased XOR mRNA expression has been shown in the lactating mammary gland of mice as compared to the non-lactating breast (Kurosaki et al., 1996). Although we initially saw no staining of epithelial cells of the large ducts in non-lactating breast tissue (II), modification of the method improved sensitivity and allowed detection of XOR in these cells (IV).

2.2. Vascular endothelium

In the present study, XOR was localized to the cytoplasm of the capillary endothelial cells of the intestine, mammary gland, skeletal muscle, and kidney. Previous data have also shown XOR expression in capillary endothelial cells of human skeletal muscle (Hellsten-Westing, 1993), as well as bovine mammary gland, intestine, and skeletal muscle (Jarasch et al., 1981). The low XOR mRNA concentrations previously detected in the human kidney (Saksela et al., 1998) can be explained by expression of the protein only in the vascular compartment of this organ. Such XOR-rich but relatively small subpopulations of endothelial cells could account for the minimal XOR activities detected in homogenates of human muscle (Hellsten-Westing, 1993) and kidney (Saksela et al., 1998).

Although vascular endothelial cells may produce XOR themselves, the possibility that circulating XOR adheres to or is taken up by the endothelium cannot be ruled out without data showing XOR mRNA expression in these cells. XOR activity and/or protein have been detected in serum after certain clinical conditions, such as intestinal ischemia (Terada et al., 1992a), liver transplantation (Pesonen et al., 1998), and viral hepatitis (Battelli et al., 1999). Once in the circulation, XOR has the capacity to bind to glycosaminoglycans on the surface of vascular endothelial cells (Adachi et al., 1993; Houston et al., 1999), and subsequently to migrate to an intracellular compartment by endocytosis (Houston et al., 1999).

The vascular endothelium comprises a heterogenous cell population which is specialized, in response to genetic programs and micro-environmental signals, to take distinct roles in different vessels, tissues, and organs (Chi et al., 2003; Risau, 1995). This may be the reason for the selectivity of XOR expression in the capillary endothelium and arterioles of certain organs only.

2.3. Heart and brain

Trace amounts of XOR activity in the human heart (Wajner and Harkness, 1989) as well as mRNA in the human heart and brain (Saksela et al., 1998), have previously been detected. In contrast, the results in this study suggest that the human heart and brain parenchyma, and also the respective vascular compartments are devoid of XOR. This is of particular importance, since XOR has been suggested to have a role in the pathogenesis of cell damage following ischemia-reperfusion of the heart (Das et al., 1987). For example, in the isolated postischemic rat heart, free radical generation was shown to be mediated by XOR (Xia and Zweier, 1995). However, most of the evidence is indirect and may at least partly be accounted for by pharmacological effects of allopurinol and its metabolite oxypurinol as free radical scavengers, not dependent of XOR inhibition (Moorhouse et al., 1987). Our results showing lack of XOR in heart and brain does not exclude the possibility that XOR is induced during pathological conditions.

2.4. Methodological aspects

Contradictory results in studies on XOR expression in human and animal organs may partly be explained by methodological problems. The specificity of the antibodies is crucial, and cross-reaction with the closely related AO protein (Terao et al., 1998) is a distinct possibility. Although we cannot strictly rule out this potential problem, it is rendered unlikely by the negative staining of the lung which is known to strongly express AO (Li Calzi et al., 1995).

The specificity of our immunohistochemical findings was confirmed by staining the sections with and without the rabbit preimmune serum. Furthermore, the sections were stained with anti-XOR antibodies after absorption with purified XOR. Every immunohistochemical method has a limit of sensitivity, and positive staining indicates that this limit has been exceeded. A negative result does not rule out the presence of minute amounts of protein.

The mRNA for XOR could not be detected using *in situ* hybridization, because the sensitivity of the assay apparently was not sufficient.

3. Regulation of XOR by oxygen (III)

3.1. Hypoxia

Our results indicate that oxygen post-translationally inactivates the molybdenum center of the XOR enzyme and that reactivation occurs in hypoxia. Previously, post-translational increase in XOR activity in hypoxic cells has been suggested to be a consequence of XO to XDH conversion (Wiezorek et al., 1994). In contrast, we could not show a change in the XDH to XO ratio

during hypoxia. However, our data are in line with those showing an increase in both enzyme forms (Hassoun et al., 1994; Terada et al., 1992b). In concordance with our findings, one study showed increased XOR activity but unchanged XOR protein and mRNA concentrations in anoxic bovine endothelial cells (Poss et al., 1996). However, others have concluded that XOR is transcriptionally regulated in hypoxia (Partridge et al., 1992; Terada et al., 1997). Based on our results, the suggestion that the increased XOR activity in hypoxia may be due to enhanced phosphorylation of the enzyme after 4 h of exposure in hypoxia (Kayyali et al., 2001) cannot be ruled out, although we could not detect activation of XOR during the same time frame.

Lack of induction of XOR activity after treatment of cells with cobalt chloride suggests that the human XOR gene is not HIF-1α responsive, also supporting a role for post-translational rather than transcriptional regulation of human XOR in hypoxia, at least in the cell model we used.

3.2. Hyperoxia

Previous reports have suggested that XOR is transcriptionally downregulated in hyperoxia (Hassoun et al., 1994; Lanzillo et al., 1996). Our results indicating post-translational inactivation of XOR in hyperoxia do not support the mechanistic interpretation of these data, whereas they are in line with findings showing that reactive oxygen species inactivate the purified enzyme (Terada et al., 1991) and rat endothelial cell XOR (Terada et al., 1988). We suggest that XOR is inactivated during hyperoxia by reactive oxygen species, which convert the molybdenum center of the enzyme reversibly into an inactive desulfo-form. It is of interest to note that the free radical nitric oxide is also capable of reacting with the essential sulfur of the molybdenum center of XOR, to produce inactive desulfo XOR (Ichimori et al., 1999).

We showed that the FAD center is unaffected in normoxic, hypoxic, and hyperoxic cells, but since we did not study the function of the Fe/S clusters which are potentially sensitive to oxygen (Beinert et al., 1997), we cannot rule out that these were also altered in the hyperoxic cells.

Discrepancies in the published reports concerning the regulation of XOR by oxygen may reflect cell, tissue and/or species-specific variations or differences in experimental conditions. We analyzed the regulation of XOR in hypoxia and hyperoxia in human cells, using transformed bronchial epithelial cells. Because of their anatomical localization, lung-derived cells are in direct contact with oxygen *in vivo,* while XOR operates under a much lower oxygen tension in most other organs (Ganong, 2003), including liver (Wolfle et al., 1983) and breast (Vaupel et al., 1991) tissue. Thus, it is possible that the results obtained cannot be extrapolated to other cell types and organs, or across species barriers. Nevertheless, it is worth pointing out that the so-called normal atmosphere in cell culture (21% O_2) actually represents hyperoxia, and proteins other than XOR may also be sensitive to oxygen-induced damage.

4. XOR expression in breast cancer (IV)

4.1. Expression and prognostic value of XOR in breast cancer

We studied the expression of XOR in a large series of human breast cancer. The nationwide FinProg breast cancer database and the accompanying tumor tissue microarrays, containing specimens from approximately 1930 patients, were available for immunohistochemical analysis of XOR expression. Our results showed that the XOR expression in the cytoplasm of the epithelial cells was decreased in more than half of the breast tumors studied. This is in line with previous studies using animal tissues showing XOR activity to be lowered or absent in hepatomas (Ikegami et al., 1986)

and colon tumors (Weber et al., 1978) as compared to corresponding normal tissue.

Cytoplasmic XOR expression was relatively weakly associatiated with tumor size, nodal status, histologic grade, and no association was found between cytoplasmic XOR and hormone receptor status, proliferation rate as measured by Ki-67, p53 protein expression, or *ERBB2* amplification.

Lack of cytoplasmic XOR was not restricted to certain types of tumors, and XOR was also downregulated in early stage tumors, i.e. tumors of small size, node negative tumors, as well as p53 negative and estrogen receptor positive breast cancer, and those with low Ki-67 expression. This finding could indicate that downregulation of XOR is an early event in tumorigenesis, which is supported by the observation that XOR is also downregulated in ductal *in situ* carcinoma as compared to normal breast epithelium.

Nuclear XOR expression has not been reported previously. XOR was expressed in a small fraction (5%) of the epithelial cell nuclei in a minority of the normal breast samples studied. In contrast, moderate to high nuclear XOR expression was found in approximately half of the breast cancers. Presence of XOR in cancer cell nuclei was associated with some adverse prognostic features, including increased COX-2 expression, a large tumor size, and *ERBB2* amplification, but not with survival. The mechanism of translocation and the functions of nuclear XOR, if any, remain to be elucidated although unspecific binding of anti-XOR antibodies to the nucleus or nuclear membrane cannot be ruled out.

The use of a tissue microarray technique (Kononen et al., 1998) enables evaluation of a large number of samples providing power to the statistical analysis. Decreased cytoplasmic XOR expression was found to be an independent prognostic factor in breast cancer, also in a subgroup of patients with small tumors and axillary node-negative disease. Thus, XOR could potentially emerge as a clinically useful marker in identifying patients with high risk of distant recurrence, even among those patients exhibiting an otherwise favorable prognostic profile according to established prognostic criteria e.g. patients with a small tumor size and lymph-node negative disease.

4.2. Biological implications

The results presented in (IV) will have to be validated in another series of breast cancer and a prospective study should be performed to confirm our results. Measurement of XOR activities and mRNA concentrations in breast cancer specimens will allow an interpretation of the present results based upon immunohistochemistry. It will also be important to elucidate the regulation of XOR during breast tumorigenesis and to clarify, whether the down-regulation of the purine catabolic XOR is associated with a corresponding up-regulation of purine anabolic enzymes in the same tumors. The role of other milk secretion-related proteins in breast cancer should also be analyzed, since these have been shown to be co-localized with XOR during secretion of the milk fat droplet in lactating mammary epithelial cells (McManaman et al., 2002). Moreover, it will be interesting to clarify whether XOR is also down-regulated in other tumors derived from cells with strong normal (wild-type) expression of XOR, such as intestinal epithelial cells and hepatocytes.

The concentration of a protein can potentially be regulated at several levels, particularly by transcriptional or posttranslational modification. Genetic alterations, such as allelic losses in the XOR chromosomal region, may also explain the diminished XOR expression. Such inactivating mutations include point mutations and deletions (Lengauer et al., 1998), leading to the loss of one allele detectable as loss of heterozygosity (LOH). Regions of the genome showing numerous LOH are expected to harbor tumor suppressor genes causing loss-of-function

effects. Also, “epigenetic” modifications such as hypermethylation of the promoter region should be taken into consideration (Herman and Baylin, 2003). To elucidate whether the diminished XOR expression is due to silencing of the XOR gene, LOH and comparative genomic hybridization analysis would be informative.

It remains to be explored, whether tumors lacking XOR respond differently to pharmacological and hormonal therapy compared to those showing XOR expression, and if XOR expression status predicts response to a specific therapy.

CONCLUSIONS

In this series of studies, the molecular forms of XOR, the relationship between XOR expression and XOR activity, and the expression of the protein in normal human tissues were elucidated. Furthermore, the regulation of the enzyme by oxygen in cultured human cells, and the expression of XOR and its potential prognostic significance in breast cancer were evaluated. Based on the results presented in this thesis, the main conclusions are:

1. Human XOR has a similar molecular size as other mammalian XOR enzymes. Since a linear correlation between XOR activity and XOR protein was found in the liver and intestine during development, the amount of inactive XOR apparently is small (I), whereas in human milk only approximately 20% of XOR appears active.

2. In the normal human liver XOR is predominantly localized to periportal hepatocytes, and in the proximal intestine to enterocytes and goblet cells. The epithelial cells of the lactating mammary gland express XOR strongly but the epithelial cells of the normal mammary gland only moderately. Capillary endothelial cells in the intestine, mammary gland, and skeletal muscle show XOR immunoreactivity, whereas all cells in the heart, brain, and lung are below the detection limit (II).

3. Hyperoxia, even under "normal" cell culture conditions, inactivates the molybdenum center of XOR posttranslationally, leading to loss of enzyme activity. The inactivation is reversed in hypoxia, resulting in apparent enzyme induction (III).

4. The expression of XOR is decreased or undetectable in more than half of the cases of breast cancer, as compared to normal breast epithelium. Decreased expression of the protein in the cytoplasm is associated with several adverse prognostic features, but not with estrogen and progesterone receptor status, tumor suppressor protein p53, or proliferation associated Ki-67 antigen. Lack of XOR protein has independent prognostic value both in the entire patient series and in the subgroup of axillary-node negative disease (IV).

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