A novel type of glutathione peroxidase: expression and regulation during wound repair

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We have previously identified and cloned a novel keratinocyte growth factor (KGF)-regulated gene in human keratinocytes that encodes the human homologue of a bovine non-selenium glutathione peroxidase (GPx). To gain insight into the regulation of this gene *in vivo*, we isolated the murine homologue from a mouse skin cDNA library. *In vitro* transcription/translation demonstrated that the cDNA encodes a 27 kDa protein. Furthermore, we amplified by PCR a partial cDNA that most likely corresponds to a related gene. RNase protection analysis revealed tissue-specific expression of both genes and the occurrence of alternative splicing or RNA editing of at least one of the primary

transcripts. Similar to that of KGF, expression of GPx was strongly induced after cutaneous injury, and each isoform displayed unique kinetics of expression during the repair process. *In situ* hybridization studies demonstrated high levels of GPx mRNA in keratinocytes of the hyperproliferative epithelium at the wound edge. Since these cells express functional KGF receptors, induction of GPx expression by KGF might also occur *in vivo*. These data suggest a role for GPx in the protection of epithelial cells against oxidative stress, particularly during the inflammatory phase of wound repair.

INTRODUCTION

Due to its accessibility, the skin is permanently exposed to multiple harmful environmental influences, such as UV irradiation and noxious xenobiotics. Many of these induce the generation of reactive oxygen species (ROS), such as hydrogen peroxide, organic hydroperoxides and oxyradicals. ROS are frequently intermediates and (by-)products of numerous chemical reactions, e.g. redox reactions and homolytic molecular cleavages. In addition, endogenous ROS are generated in all cell types in the course of a broad variety of common metabolic processes, e.g. in the respiratory chain. Particularly large amounts are produced in inflamed tissues, where they are released by activated macrophages during the respiratory burst that represents a defence mechanism against microbial infection [1]. Since, on the one hand, excessive accumulation of ROS is extremely toxic and under some circumstances can even lead to neoplastic transformation [2], but, on the other hand, a basal level of these species is needed for survival in some cell types [3], the intracellular concentration of ROS has to be tightly regulated. In general, there are two major strategies that convey partial resistance against oxidative stress to most cell types: (1) small antioxidant molecules such as ascorbate, polyunsaturated fatty acids and sugars (mainly mannitol), and (2) ROS-scavenging enzymes, such as superoxide dismutases, catalase and various peroxidases [4]. Epidermal cells have been shown to possess all of these standard defence mechanisms against ROS [5]. As these cells are especially prone to oxidative stress, one could speculate about additional strategies that they might employ.

We have identified novel genes that are regulated by keratinocyte growth factor (KGF) in cultured human keratinocytes. KGF is a member of the fibroblast growth factor family, and is a potent and specific mitogen for epithelial cells [6,7]. One of

these KGF-regulated genes encodes the human homologue of a previously identified glutathione peroxidase (GPx) [8]. GPx enzymes use the tripeptide glutathione to reduce hydrogen peroxide and a multitude of organic hydroperoxides. They represent a large and, with respect to tissue distribution and intracellular localization, heterogeneous group of functionally similar enzymes [9]. Nevertheless, our novel protein displayed only low sequence identity with the various known members of this family, such as the tetrameric cytosolic selenoenzyme [10,11], the membrane-bound selenoenzyme phospholipid hydroperoxide GPx [12], the glycosylated extracellular enzyme [13], and the Alpha and Theta subclasses of glutathione S-transferases, which also exhibit GPx activity [14]. However, it was strikingly similar to a novel GPx from bovine ciliary body, of which only a 29-amino-acid N-terminal peptide had been sequenced [15]. GPx expression was induced by KGF, but not by serum growth factors or pro-inflammatory cytokines [8]. Since KGF expression by dermal fibroblasts is dramatically up-regulated during the course of cutaneous wound repair in mice and humans [15,16], we speculated about a possible induction of GPx by KGF during the early inflammatory phase of wound healing, which would bring about additional resistance to ROS generated during the respiratory burst of leucocytes.

In order to study the regulation of GPx expression during wound repair, we isolated the murine homologue of our novel gene from a cDNA library. In the present study, we show that GPx expression is up-regulated to a large extent during the early inflammatory phase. We further provide evidence that GPx-overexpressing cells are predominantly located in the hyperproliferative epithelium at the wound edge. These cells have been shown to express the only known high-affinity receptor for KGF, a splice variant of fibroblast growth factor receptor 2 [16–18], suggesting that GPx overexpression in these cells might be

Abbreviations used: KGF, keratinocyte growth factor; GPx, glutathione peroxidase; mGPx, mouse non-selenium GPx; mGPx*, second isoform of mGPx; ROS, reactive oxygen species; RT-PCR, reverse transcription-PCR.

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directly related to elevated KGF levels. These data point to a novel function for this growth factor during the inflammatory phase of cutaneous wound repair.

MATERIALS AND METHODS

Animal care

Balb/c mice were obtained from the animal care facility of the Max-Planck-Institute of Biochemistry. They were housed and fed according to Federal guidelines, and all procedures were approved by the Local Government of Bavaria.

Wounding and preparation of wound tissue

Three independent wound healing experiments were performed with female Balb/c mice (8–12 weeks of age), as described in [15]. For each experiment, 23 animals were anaesthetized by intraperitoneal injection of avertin. The hair on the animals' backs was cut off with fine scissors and the skin was wiped with 70 % (v/v) ethanol. Six full-thickness wounds (6 mm diameter; 3–4 mm apart) were generated on each animal by excising the skin and *panniculus carnosus*. The wounds were allowed to dry to form a scab. At 1, 3, 5, 7 and 13 days after wounding, four animals were killed and the wounds were harvested. The complete wound, including 2 mm of the margins, was excised at each time point. A similar amount of back skin from three non-wounded animals served as control. Wounds from animals at each time point were combined, frozen immediately in liquid nitrogen and stored at -70°C until used for RNA isolation.

RNA isolation and RNase protection assay

Total cellular RNA was isolated as described [19]. For the generation of the cDNA library, polyadenylated RNA was purified from total Balb/c mouse back skin via affinity chromatography on oligo(dT)–cellulose [20] using the Pharmacia mRNA isolation kit. RNase protection experiments were carried out according to [15]. A 396 bp mouse GPx (mGPx) PCR fragment or a 213 bp mGPx* fragment (where mGPx* is a second isoform of murine non-selenium GPx) were used as templates (Table 1).

In situ hybridization

A 188 bp fragment from the mGPx open reading frame (Table 1) was subcloned into pBluescript KSII(+) (Stratagene) and linearized. Sense and antisense riboprobes were generated using T3 or T7 RNA polymerases and [α - ^{32}S]UTP. Paraffin-embedded sections (6 μm) were hybridized as described in [22], with the exception that hybridization was carried out at 50°C and the high-stringency wash was performed at 55°C . After hybridization, sections were coated with NTB2 nuclear emulsion (Kodak) and exposed at 4°C in the dark for 4 weeks. After development, sections were counterstained with haematoxylin/eosin.

Reverse transcription–PCR (RT–PCR)

A 5 μg sample of total mouse tail skin RNA was reverse-transcribed using an oligo(dT)₁₅ primer (Promega). Subsequently, 30 PCR cycles were carried out at an annealing temperature of 52°C . Four different sets of primers, derived from different parts of the human GPx sequence (accession number D14662 in the EMBL database), were used: primer 5'1, 5'-TGGGGCATTCTCTTCTCCAC-3' (nt 140–60 of the human and nt 165–185 of the murine sequence); primer 5'2, 5'-CTGGCACCAGAATTGCCAAAG-3' (nt 212–232/237–257); primer 3'1, 5'-CATGCCCTTTTCATCCTTCTC-3' (nt 424–404/449–429); primer 3'2, 5'-CATCACACTATCCCCATCCTT-3' (nt 607–587/632–612). PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. After purification, they were ligated into pBluescript KSII(+) (Stratagene).

Southern blot analysis

A 10 μg sample of chromosomal DNA [23] was digested with *Bam*HI, *Eco*RI or *Hind*III restriction enzymes, subjected to gel electrophoresis on vertical 1 % (w/v) agarose gels and transferred to Gene Screen Plus nylon membranes (NEN, Boston, MA, U.S.A.). Hybridization was carried out overnight at 42°C in 50 % formamide/5 $\times$ SSC using 2×10^7 c.p.m. of ^{32}P -labelled 396

Table 1 Overview of all DNA templates described in this study

A summary of all DNA templates described in this study is given. The putative mGPx open reading frame is located between nucleotides 69 and 740.

Fragment	Generation	Nucleotide positions relative to full-length mouse cDNA	Size (bp)	Used for
mGPx 396 bp	PCR with primers 5'2 and 3'2 and cDNA derived from mouse tail skin	237–632	396	Library screen; Southern analysis; (Northern analysis); RNase protection analysis
mGPxS 396 bp	PCR with primers 5'2 and 3'2 and cDNA derived from mouse intestine and stomach	237–632 with two nucleotide exchanges at positions 393 and 439	396	
mGPx*	PCR with primers 5'2 and 3'2 and cDNA derived from mouse tail skin	237–449; approx. 90 % identity	213	RNase protection analysis
mGPx 188 bp	<i>Bam</i> HI digest of mGPx 396 bp	237–424	188	<i>In situ</i> hybridization
mGPx full-length	Library screen	1–1470	1470	<i>In vitro</i> transcription/translation

bp mGPx PCR fragment (Table 1) as a probe. Radioactive labelling was performed as described in [21] using the Rediprime DNA labelling system (Amersham Life Science). Labelled fragments were purified by gel filtration on Sephadex G-50 columns (Pharmacia).

Generation of a mouse skin cDNA library and molecular cloning of mGPx

A phage λ mouse (Balb/c) back skin cDNA library was constructed using the Stratagene λ -ZAP cDNA synthesis kit. In a first attempt, 3×10^5 plaques were screened using the ^{32}P -labelled 396 bp mGPx PCR fragment (Table 1) as a probe; 12 positive plaques were re-screened in the same way. Single cDNA clones were used for *in vivo* excision and sequenced.

In vitro transcription and translation

A 1 μg sample of the *in vivo* excised plasmid pBluescript SKII-full-length mGPx (Table 1) was used as a template in a coupled reticulocyte lysate *in vitro* transcription/translation system (Promega) using T3 RNA polymerase and [^{35}S]methionine (1000 Ci/mmol) at 10 mCi/ml (Amersham). Proteins were separated on 15% (w/v) polyacrylamide gels under denaturing conditions. Radiolabelled proteins were subsequently analysed by autoradiography.

RESULTS

Molecular cloning of mGPx

In order to analyse possible functions of GPx *in vivo*, we first cloned the cDNA of the murine homologue of this gene. In a first attempt, we generated single-stranded cDNA from murine tail skin. Subsequently, using primers corresponding to the human

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CGCAGGAGCCCGCCCGCTGCTGCTGCTGCGGCTGCGCCTCCTTGTCTTC 50
AGCGTCACCACTGCCGCCATGCGCCGAGGGTTGCTTCTCGGGGACGAAGC 100
CCCCAACTTTGAGGCCAATACCACCATCGGCCGATCCGCTTCCAGGATT 150
TCCTGGGAGATTTCATGGGGCATTCTCTTTCCCAACCCAGGGACTTTACC 200
CCAGTGTGCACCAAGAGTCTGGGAGAGCTGCAAGCTGGCGCCAGAGTT 250
TGCCCAAGAGGAATGTTAAGTTGATGCTCTTTCAATAGACAGTGTGAGG 300
ATCATCTTGCTGAGCAAGGACATCAATGCTTACAATGGTGAAACACCC 350
ACGGAAGAGTTGCCATTTCCCATCATTGATGATAAGGGCAGGACCTTGC 400
CATCTCTTTGGGCATGTTGATCCAGTCGAGAAGGACGATAACAACATGC 450
CTGTGACGGCCCGTGGTGTTCATTTTGGGCCCTGACAAGAACTGAAG 500
CTGTCTATCTCTACCTGCGCCACGAGGCGAGCACTTTGATGAGATTCT 550
CAGAGTGGTTGACTCTCTCCAGCTGACAGGCACAAAGCCGGTTGCCACCC 600
CAGTTGACTGGAAGAAGGGAGAGAGCGGTGATGGTAGTTCCCAACCTCTCC 650
GAAGAGGAAGCCAAACATGTTTCCCTAAAGGAGTCTTACCAAGAGAGCT 700
CCCGTCTGGCAAAAAATACCTCCGTTATACACCCAGCCTTAAAGTCTT 750
CGGAAATGGGGCTGCATCTGCACATCCAGTACTGGGGCTGAGGTAGTC 800
AGCTGGCAGCCGTGGTCTTGCAGGAGGTCGCTAGAAAGATCGTGGCAT 850
GATCAGAGCCGCTCTGATAGTCGCTGCTATCTACTGGGTCATTAAAT 900
GGAAATGGCACCAAAACCTTCTCGGGATTCTTTACTCTGTGCCTTCGCCA 950
GCATTTGCCCTCTGCTGTCAAGTGCCTACTGACTGGCTCTCTTTG 1000
AAACGAATTATGATTGAAGATTCTTAGGTCTCTGCAGGGTCTTTGATC 1050
AGCAAAACAGGTAGTGTGAGTGTGGGCTCTGTGCTAGAAATGATGAACAC 1100
CTTTGTATCTTTCCGAAGTGAATCTCTGTTACCCATTTTGGAGAGCAC 1150
TGACATAGGGAGAAGCTTTCGATTCTGTATTTTGTAAATAAAAGTGG 1200
GGACAGCCGGGAGAAATCTTACAGGGAATCTATTGTAAGTTCTATCGAA 1250
GTGGGCTCAGAAAGCCTTTCCGCTCCCAAGAGTGCGCATGTACCTCCTAG 1300
AGTTTCCACATCTGCTCTCTGGTGTGCTGCTGTGAACGCACCTTATA 1350
AAAGACGGGCGGTGACAGTGTTTTACCACTCAGTGTCTAGTAGTGGGTG 1400
GCCATTCTGAATCTGCTTTTGTAGGTTCAACAAATAAAATCCTGATCA 1450
GAAAAAAAAAAAAAAAAA
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Figure 1 Complete cDNA sequence of mGPx

The putative start and stop codons are shown in bold type. The putative open reading frame is underlined.

huGPx	MPGGLLLGDEVAPNFEANTTVGRIRFHDGLDGSWGLFESHPRDFTPVCTTE
mGPx	<u>MPGGLLLGDEAPNFEANTTIGRIRFHDGLDGSWGLFESHPRDFTPVCTTE</u>
bGPx (a)	PGGLLLGDEAPNFEANTTIGGISFXXXLG ? ?
huGPx	LGRAAKLAPEFAKRNVKLIASIDSVEDHLAWSKDINAYNCEEPTKLPF
mGPx	<u>LGRAAKLAPEFAKRNVKLIASIDSVEDHLAWSKDINAYNGETPTKLPF</u>
mGPx*	<u>RNVQLIALPVDSDVHLAWSKDINAYS</u> GTAPTEKLPF
huGPx	PIIDDRNRELAILLGMLDPAEKDEKGMFVTARVVFVFGPDKKLKLSILYP
mGPx	<u>PIIDDKGRDLAILLGMDFVEKDDNNMPEVTARVVFIFGPDKKLKLSILYP</u>
mGPx*	<u>PIIDDEDRDLAILLGMDFV</u>
mGPxS	N A
huGPx	ATTGRNFDEILRVVISLQLTAEKRVATPVDWKDGSVMVLPETIPEEAAKK
mGPx	<u>ATTGRNFDEILRVVDSLQLTGTKPVATAVDWKKGESVMVVPETLSEEEAKQ</u>
huGPx	LFPKGVFTKELPSGKKYLRYTPQP
mGPx	<u>CFPKGVFTKELPSGKKYLRYTPQR</u>

Figure 2 Alignment of the amino acid sequences of human, bovine and mouse GPx enzymes

A comparison of the amino acid sequences deduced from human (huGPx) and mouse cDNA clones with the N-terminal GPx peptide fragment from bovine ciliary body (bGPx) is shown. The question marks indicate positions of less confidence [15]. The sequence of the full-length mouse clone is underlined. The two amino acid substitutions occurring in the putative splice variant (mGPxS) that is expressed in intestine, stomach and liver are indicated. Deviating positions are shown in bold type.

sequence, several fragments of 200–450 bp were amplified by PCR. A 396 bp PCR fragment that we had amplified with primers 5'2 and 3'2 was radiolabelled and served as a probe for screening of a cDNA library that we had generated from mouse back skin. The longest cDNA insert that we obtained (1470 bp; sequence shown in Figure 1) is likely to encode the full-length GPx protein, since we detected a 1.5 kb mRNA by hybridizing a Northern blot of murine skin with the same 396 bp probe (results not shown). The deduced amino acid sequence of the full-length mGPx protein is shown in Figure 2. Compared with the N-terminal fragment of bovine GPx (29 amino acids), this protein differed in only two positions. Compared with the human protein, mGPx was approx. 90% identical (Figure 2), demonstrating that we had indeed cloned the murine homologue of our KGF-regulated gene.

mGPx is a protein of approximately 27 kDa

To determine the size of the mGPx protein and to confirm the putative open reading frame of 224 amino acids, we performed *in vitro* transcription/translation experiments using our full-length cDNA clone as a template. The resulting protein was labelled with [^{35}S]methionine and analysed by SDS/PAGE followed by autoradiography (Figure 3). The labelled protein had an approximate molecular mass of 27 kDa, which almost corresponds to the calculated molecular mass of 25 kDa. The additional three bands appearing at slightly higher molecular masses might be the result of post-translational modifications other than glycosylation, since rabbit reticulocyte lysate does not contain the microsomal fraction. Alternatively, the unmodified protein could possibly migrate at 30 kDa. The three smaller

Figure 3 *In vitro* transcription/translation

The full-length mouse cDNA clone was used as a template for *in vitro* transcription/translation using a coupled reticulocyte lysate system. Translation products were labelled with [³⁵S]methionine, separated on a 15% polyacrylamide gel and analysed by autoradiography (lane 2). Lane 1 represents the negative control, in which transcription/translation was carried out without a template. Positions of molecular mass markers (kDa) are shown on the left.

Figure 4 mGPx is a member of a multigene family

A Southern blot analysis of mouse and rat genomic DNAs that had been digested with *Bam*HI, *Eco*RI or *Hind*III restriction endonucleases is shown. Multiple bands were detected under low-stringency conditions, whereas most bands disappeared after a high-stringency wash. The remaining bands are marked by arrows in the three lanes containing the mouse DNA.

proteins might then be a result of preliminary transcriptional or translational termination or of partial proteolytic degradation.

GPx is a member of a multigene family

To determine whether mGPx is a unique gene or a member of a multigene family, we performed low-stringency Southern blot experiments with mouse and rat genomic DNA. The 396 bp mGPx PCR fragment that had also been used for the screening of the cDNA library was used as a hybridization probe. The complex pattern of bands that was observed under low-stringency conditions (Figure 4) indicates that mGPx and rat GPx might be members of widespread multigene families. Indeed, using low-stringency RT-PCR with RNA derived from mouse tail skin, we identified a partial cDNA clone (mGPx* in Figure 2) that was only 90% identical to the full-length cDNA at the nucleic acid (results not shown) and protein (Figure 2) levels. These significant differences suggest that the GPx* mRNA is derived from a distinct but related gene.

Figure 5 Differential expression of mGPx in mouse tissues

(A) Portions of 30 µg of total RNA from different mouse tissues were analysed by RNase protection assay. The 396 bp mGPx fragment obtained by RT-PCR was used to generate an antisense riboprobe. The longest protected fragment was generated by mRNA corresponding to our PCR fragment. The two smaller protected fragments seen in brain, intestine, stomach and liver were generated by mRNA corresponding to a splice or editing variant. The film was exposed for 16 h. (B) Tissue distribution of mGPx* analysed by an RNase protection assay. The 213 bp mGPx* PCR fragment was used as a probe. Portions of 30 µg of RNA were used; the film was exposed for 4 days.

Figure 6 mGPx expression is strongly induced after skin injury

The results of an RNase protection assay using samples of 30 µg of total RNA from murine wounds are shown. The time after wounding (d, days) is indicated above each lane. The 396 bp PCR fragment (see Figure 5A) was used for the generation of a ³²P-labelled antisense riboprobe. The hybridization probe (1000 c.p.m.) was loaded in the lane labelled 'Probe' and used as a size marker. A 30 µg portion of tRNA was used as a negative control. The film was exposed for 16 h.

GPx is expressed in a tissue-specific manner

To determine the tissue-specific expression of mGPx and mGPx*, we performed RNase protection assays with RNAs from different mouse tissues. The 396 bp mGPx PCR fragment that had also been used for library screening and Southern blot analysis was used as a template for the generation of a ³²P-labelled antisense riboprobe. As shown in Figure 5(A), GPx expression was high in heart, kidney and liver, moderate in brain and stomach, and barely detectable in the intestine. In intestine, stomach and liver, we detected two smaller protected fragments instead of the full-length one. Since every single mismatch can be detected by RNase protection assay under our high-stringency conditions, the generation of these shorter bands suggested the existence of another RNA variant in these tissues. To test this possibility, we amplified the same 396 bp region from cDNAs derived from these tissues by RT-PCR and thereby indeed determined the

Figure 7 mGPx is expressed in keratinocytes at the wound edge

A full-thickness excisional wound was isolated 5 days after wounding, bisected, fixed in 4% paraformaldehyde and embedded in paraffin. Sections of 6 μ m from the middle of the wound were hybridized with a 188 bp antisense riboprobe for mGPx. GPx is predominantly expressed by keratinocytes of the hyperproliferative epithelium at the wound edge. Note the cut-off between highly expressing epidermis at the wound edge and non-expressing epidermis further back (**A**). The silver grains produced by the radioactive probe appear as bright dots in the dark-field survey (**A** and **B**) and as black dots in the bright-field survey (**C**). The slides were exposed for 4 weeks. Abbreviations: D, dermis; Es, Eschar; HE, hyperproliferative epithelium; H, hair follicle. Magnification: (A and B) $\times 85$; (C) $\times 170$.

presence of two nucleotide exchanges at the locations predicted by the sizes of the smaller protected fragments. These two RNA variants might be a result of alternative splicing or RNA editing.

In all tissues tested, expression levels of the related gene mGPx* were much lower than for GPx. Furthermore, the tissue-specific expression pattern was slightly different for this gene (Figure 5B). mGPx* transcripts were most abundant in the liver, followed by kidney, heart and brain. By contrast, expression of this gene was not detectable in intestine and stomach.

Expression of mGPx is strongly induced during wound healing

To determine if GPx induction by KGF might be of physiological importance, we analysed mGPx expression under conditions where KGF levels are high, i.e. cutaneous wound repair. We have demonstrated a more than 100-fold induction of KGF expression within 24 h after injury. Expression of KGF subsequently declined, but high levels of KGF mRNA were found during the first 7 days of the repair process [16]. We have used the same wound-healing model to determine expression of mGPx. Figure 6 shows an RNase protection assay using the same probe as in Figure 5(A). As deduced from the exposure time of the autoradiography, expression of both GPx variants was low in normal skin. However, GPx levels increased significantly after injury. Furthermore, the kinetics of one variant (middle and bottom bands in Figure 6) corresponded to the time course observed for KGF expression [16]. In contrast, the mRNA levels of the other variant (top band in Figure 6) decreased rapidly after the initial induction. These different kinetics indicate that not only expression of the mGPx gene but also the splicing or editing pattern of the corresponding transcripts might be regulated. In addition to mGPx, mGPx* was also expressed during wound healing, although at much lower levels. Interestingly, the time courses of expression of this gene and of one of the mGPx variants (bottom band in Figure 6) were comparable (results not shown). Furthermore, this correlated with the time course of KGF expression during wound healing [16].

GPx-expressing cells are located within the hyperproliferative epithelium at the wound edge

To locate GPx-expressing cells within the wound, *in situ* hybridization studies were performed. The specificity of the signals was confirmed by hybridization with a sense riboprobe (negative control; results not shown). As shown in Figure 7, mGPx transcripts were highly abundant in keratinocytes of the hyperproliferative epithelium at the wound edge, which are known to express functional KGF receptors [16,17]. Under the chosen conditions, the mGPx probe is likely to detect all GPx variants, and presumably also GPx* mRNA, demonstrating that the majority of GPx and GPx* transcripts are located in the hyperproliferative epithelium. In contrast, expression of this gene was low in normal epidermis at a distance from the wound, and was barely detectable in the dermis.

DISCUSSION

Cutaneous wound repair is a complex process comprising proliferation, differentiation, necrosis and apoptosis of various cell types, and therefore has to be tightly co-ordinated [24]. This regulation is achieved primarily by a multitude of different growth factors and cytokines. Previous studies from our laboratory have demonstrated a particularly important role for KGF and its receptor in this complex interplay. Thus KGF expression by dermal fibroblasts is strongly induced as early as

24 h after skin injury [16], and expression of a dominant-negative KGF receptor in keratinocytes of transgenic mice resulted in a significant delay in wound re-epithelialization [25].

To gain insight into the mechanisms of KGF action in the skin, we set out to identify genes that are regulated by KGF in keratinocytes. Using differential display RT-PCR technology, we cloned a novel gene that encodes the human homologue of a previously identified bovine GPx [8]. To determine if GPx induction by KGF might also occur *in vivo* during wound healing, we first isolated the murine homologue from a skin cDNA library. The sizes of the transcripts and the encoded proteins are similar for the mouse and human proteins. Furthermore, the longest open reading frames of the human and mouse cDNAs were approx. 90% identical at the nucleic acid and protein levels, suggesting that we had indeed cloned a murine homologue of our KGF-induced gene.

Nevertheless, several results indicate that there may be even more mGPx genes. In addition to our full-length cDNA clone, we obtained a second partial cDNA (mGPx*) by low-stringency PCR. This 213 bp fragment differed in approx. 10% of all positions from the full-length clone as well as from the human homologue. Furthermore, a complex pattern of bands was detected by Southern blot analysis under low-stringency conditions with genomic DNA from mice and rats (the present study) and also from humans [8]. Expression of GPx and GPx* occurred in a tissue-specific manner and with different kinetics during wound repair. These findings indicate that the different isoforms might be functionally distinct. Nevertheless, the presence of at least one isoform in every tissue tested strongly suggests that GPx might be involved in central, if not essential, processes of cellular metabolism.

Using RT-PCR with RNAs from different tissues, we identified two fragments that differed in only two positions out of 396 bp, and therefore are unlikely to be derived from different genes. Both nucleotide exchanges should lead to amino acid substitutions. Consequently, the number of GPx isoforms seems to be further augmented by alternative splicing or RNA editing. Most interestingly, the splicing or editing pattern was different at early and later stages of wound repair. Alternative splicing during wound healing has already been described for the extracellular matrix protein fibronectin [26], where the effect has been attributed to different growth factors, such as transforming growth factor β [27–29].

Since expression of human GPx is strongly induced upon KGF treatment in cultured keratinocytes, we analysed mGPx expression during wound repair when KGF levels are high, i.e. cutaneous wound repair. KGF is expressed in the mesenchyme below the wound and at the wound edge [16], and acts in a paracrine manner to stimulate proliferation of epithelial cells. In the present study, we have demonstrated a strong and rapid induction of mGPx expression after injury, suggesting an important function for the enzyme during the repair process. Interestingly, GPx mRNA was particularly abundant in the keratinocytes of the hyperproliferative epithelium at the wound edge, which express the only known high-affinity receptor for KGF [16–18], suggesting that induction of GPx expression by KGF might also occur *in vivo*. Furthermore, the time course of KGF expression after injury [16] was correlated with the kinetics of mGPx and mGPx* expression.

The rapid induction of all isoforms after injury suggests that GPx might exert its function predominantly during the early inflammatory phase of wound healing. This period is characterized by the release of serum growth factors and pro-inflammatory cytokines, which attract a multitude of inflammatory cells such as macrophages and polymorphonuclear leucocytes. These cells

contain a broad variety of specialized oxidoreductases such as myeloperoxidase and, upon activation, produce numerous ROS. This phenomenon, often described as the respiratory burst, has been implicated in the defence against microbial infection and is therefore beneficial [1,30,31]. Furthermore, other cells such as fibroblasts can be stimulated to produce ROS by pro-inflammatory cytokines, such as interleukin-1 or tumour necrosis factor α [32]. However, increased levels of ROS might also have a series of negative effects. Even at low concentrations, hydrogen peroxide has been shown to inhibit keratinocyte migration and proliferation [33]. Since both processes are stimulated by KGF, the induction of a peroxidase by KGF might be a novel mechanism of KGF action. Most importantly, prolonged exposure to high concentrations of these reactive species can lead to severe tissue injury, followed by apoptotic cell death [34] or even neoplastic transformation [2]. Thus a KGF-regulated ROS-scavenging enzyme such as our GPx might be an ideal tool for keratinocytes of the hyperproliferative epithelium at the wound edge, conveying additional ROS resistance to these cells during times of intensified oxidative stress.

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