Inherent growth advantage of (pre)malignant hepatocytes associated with nuclear translocation of pro-transforming growth factor $\alpha$

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The pro-peptide of transforming growth factor $\alpha$ (proTGF$\alpha$) was recently found in hepatocyte nuclei preparing for DNA replication, which suggests a role of nuclear proTGF$\alpha$ for mitogenic signalling. This study investigates whether the nuclear occurrence of the pro-peptide is involved in the altered growth regulation of (pre)malignant hepatocytes. In human hepatocarcinogenesis, the incidence of proTGF$\alpha$-positive and replicating nuclei gradually increased from normal liver to dysplastic nodules, to hepatocellular carcinoma. ProTGF$\alpha$-positive nuclei almost always were in DNA synthesis. Also, in rat hepatocarcinogenesis, proTGF$\alpha$-positive nuclei occurred in (pre)malignant hepatocytes at significantly higher incidences than in unaltered hepatocytes. For functional studies unaltered (GSTp$^-\alpha$) and premalignant (GSTp$^+\alpha$) rat hepatocytes were isolated by collagenase perfusion and cultivated. Again, DNA synthesis occurred almost exclusively in proTGF$\alpha$-positive nuclei. GSTp$^-\alpha$ hepatocytes showed an ~3-fold higher frequency of proTGF$\alpha$-positive nuclei and DNA replication than GSTp$^+\alpha$ cells. Treatment of cultures with the mitogen cyproterone acetate (CPA) elevated the incidence of proTGF$\alpha$-positive nuclei and DNA synthesis in parallel. Conversely, transforming growth factor $\beta1$ (TGF$\beta1$) lowered both. These effects of CPA and TGF$\beta1$ were significantly more pronounced in GSTp$^+\alpha$ than in GSTp$^-\alpha$ hepatocytes. In conclusion, nuclear translocation of proTGF$\alpha$ increases in the course of hepatocarcinogenesis and appears to be involved in the inherent growth advantage of (pre)malignant hepatocytes.

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Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, particularly in Asia and Africa, accounting for about 1 million deaths per year (Parkin et al., 2001). Recently, its incidence has substantially increased in Europe and the United States (El-Serag and Mason, 1999; Parkin et al., 2001). Transforming growth factor $\alpha$ (TGF$\alpha$) is one of the cytokines, causally involved in the pathogenesis of liver cancer (Kiss et al., 1997; Grisham, 2001). It is produced by hepatocytes and nonparenchymal liver cells for paracrine, autocrine and/or juxtacrine stimulation, as shown in developing, regenerating, preneoplastic and neoplastic livers of rodents and humans (Mead and Fausto, 1989; Miller et al., 1995; Grisham, 1997; Kiss et al., 1997; Yarden and Sliwkowski, 2001). Transforming growth factor $\alpha$ appears to be upregulated in all stages of liver cancer development. Single hepatocytes, infected with the hepatitis-B virus, overexpress TGF$\alpha$ due to transactivation of the TGF$\alpha$ gene by the virus (Schirmacher et al., 1996). Hepatocellular adenomas and carcinomas as well as childhood hepatoblastomas have been found to be rich in TGF$\alpha$s, leading to elevated plasma levels of this cytokine (Yamaguchi et al., 1995; Kiss et al., 1997; Grisham, 2001).

According to textbook knowledge, TGF$\alpha$s is produced as a precursor transmembrane molecule (proTGF$\alpha$s). The ectodomain of the pro-peptide may be shed from the cell surface, where it may bind to and activate the erb-b-1 receptor, that confers the growth signal via phosphorylation cascades to the nucleus (Massagué, 1990; Yarden and Sliwkowski, 2001). Considering the upregulation of TGF$\alpha$s in human malignancies, including liver cancer, hope focuses on the possible therapeutic benefit of blocking TGF$\alpha$s-evoked signal transduction on the cell surface, for example, by blockade of the receptor or of ligand–receptor interactions (Levitzki and Gazit, 1995; Mendelsohn, 1997). In a recent study, however, we have shown that hepatocytes in the intact liver and in primary culture synthesise proTGF$\alpha$s that translocates to the nucleus, where it appears to be involved in the mitogenic response of the cell (Grasl-Kraupp et al., 2002). This proposed novel pathway was induced by various different growth stimuli and is active in three different mammalian species, including humans. In mouse hepatocytes, almost all of the proTGF$\alpha$s-pos nuclei were also...
positive for erb-b-1 (Schausberger et al., 2003). Moreover, several very recent papers suggest that the erb-b receptors 1, 3 and 4 may bypass the protein phosphorylation cascades for transducing mitogenic stimuli (Lin et al., 2001; Ni et al., 2001; Offerdinger et al., 2002). Thus, there is considerable evidence of a direct action of growth factors/growth factor receptors from the EGF/erb-b receptor family in the nucleus (Wells and Marti, 2002). The question emerges whether the nuclear occurrence of proTGFz is involved in the altered growth regulation of (pre)malignant cells.

Rodent liver provides excellent tools for functional studies on hepatocarcinogenesis (Pitot, 1990; Grasl-Kraupp et al., 1997; Grisham, 1997). Treatment of rats with genotoxic carcinogens, such as N-nitrosomorpholine (NNM), induces single initiated hepatocytes that are detectable by their selective immunoreactivity for placental glutathione-S-transferase (GSTpþ cells); a considerable fraction of these cells develops to GSTpþ (pre)malignancy (Grasl-Kraupp et al., 2000). Although human HCC often do not express GSTp due to epigenetic silencing of the gene, GSTpþ lesions of rats and (pre)malignant lesions in human liver show significant similarities, such as mutations of the wnt-pathway, overexpression of TGFz, IGF-I and -II and other growth factors (Miller et al., 1995; Grisham, 1997; Yamada et al., 1999; Yang et al., 2003). Rates of replication and death of GSTpþ cells, and thus overall cell turnover, are somewhat reduced in the single-cell stage, but are significantly increased from the two-cell stage onwards (Grasl-Kraupp et al., 1997, 2000). Thus, initiation causes a change in the growth-regulatory network that becomes evident after the first replication cycle of GSTpþ cells. Treatment with tumour promoters, such as the progesterin cyproterone acetate (CPA), or increased food intake further increases cell replication in preneoplasia, which is analogous to human hepatocarcinogenesis driven by anabolic steroids or overnutrition (Schulte-Hermann et al., 1990; Grasl-Kraupp et al., 1994; Fiel et al., 1996; Nair et al., 2002). This enhanced sensitivity of liver preneoplasia towards the various growth stimuli could result from an altered uptake, production and/or processing of endogeneous growth regulatory factors by the premalignant cell compartment.

In the present study, we asked whether the nuclear occurrence of proTGFz is involved (i) in the altered growth regulation of (pre)malignant hepatocytes and (ii) in the enhanced sensitivity of these cells towards induction of DNA replication by known growth stimulators. Recently, preneoplastic rat liver cells have become available for investigation in an ex vivo culture model (Löw-Baselli et al., 2000b). We applied this model, in combination with studies on human livers, and found that proTGFz-positive nuclei increased in the course of hepatocarcinogenesis. The possible role of nuclear proTGFz for the growth advantage of (pre)malignant cells is discussed with regard to tumour-therapeutic strategies targeted at TGFz/erb-b-1 interactions on the cell surface.

**MATERIALS AND METHODS**

**Human liver samples**

Patients suffering from dysplastic liver nodules (n = 9), hepatocellular adenoma (n = 3), or HCC (n = 10) were resected; chemotherapy had not been applied before surgery. Tissue samples were immediately fixed in 10% buffered formaldehyde. Classification of liver lesions and stage of disease followed published guidelines (Edmondson and Steiner, 1954; Hermank et al., 1993; International Working Party, 1995). For further details, see Table 1. Informed consent was obtained from all patients.

**Human hepatocyte and hepatoma cell lines**

The human hepatoma cell lines HepG2 (ATCC-No HB-8065), Hep 2B2.1–7. (ATCC-No HB-8064), and WRL 68 (ATCC-No CL-48) were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in vitro at 37°C and 5% CO2 in Dulbecco’s minimum essential medium (DMEM) supplemented with 5% foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all obtained from Gibco, Life Technologies Inc., Gaithersburg, MD, USA). Once per week cells were passaged at a seed density of 1 x 10^5 cells per 25 cm2 plate. Animals and treatment Male SPF Wistar rats, about 3 weeks old, were obtained from the Institut für Versuchstierkunde und Genetik (Himberg, Austria). Animals were kept under standardised conditions and were fed powder diet (Altromin 1321N, Altromin, Lage, FRG). At 3 weeks before treatment, animals were adapted to rhythmic feeding (from 0900 to 1400 h). This procedure synchronises DNA synthesis in the liver to a single peak per day (Grasl-Kraupp et al., 1994, 2000). After adaptation, animals were treated with a single dose of NNM (Sigma, St Louis, MO, USA; 250 mg per 10 ml phosphate-buffered saline per kg body weight). Phenobarbital (PB) was admixed to the powder diet and was fed to a subgroup of rats from day 4 to 17 months post-NNM. Concentrations of PB were adjusted every 14 months to provide a daily dose of 50 mg kg⁻¹ body weight (Löw-Baselli et al., 2000b). Animals were killed by decapitation under CO2 asphyxiation. For further details, see Grasl-Kraupp et al (1994, 2000) and Löw-Baselli et al (2000a). All experiments were performed according to the ‘Austrian Guidelines for Animal Care and Protection’, which meet the standards required by the ‘UKCCR Guidelines for the Welfare of Animals in Experimental Neoplasia’ (2nd edn.) (Workman et al, 1998).

**Histology**

Human and rat liver samples, fixed in 10% buffered formaldehyde, were processed as described (Grasl-Kraupp et al, 1994, 2000;
Löw-Baselli et al, 2000a); two serial sections, 1 μm thick, were cut; one of the sections was stained for GSTp (rat only) or Ki-67 (human only), the second one was stained for TGFz.

Immunostaining for TGFz, GSTp and Ki-67 The primary antibodies used were rabbit polyclonal IgG against rat Yp-subunit of GSTp (Biotrin International, Dublin, Eire); mouse monoclonal IgG against recombinant mature TGFz encompassing amino acids 39–88 (clone 213-4-4, Oncogene Science, Uniondale, NY, USA); mouse monoclonal IgG against a synthetic peptide encompassing amino acids 144–160 of the C-terminus of GSTp (Biotrin International, Dublin, Eire); mouse monoclonal antisera against amino acids 39–88 of mature TGFβ (human only), the second one was stained for TGFβ, one of the sections was stained for GSTp (rat only) or Ki-67 (human only), the second one was stained for TGFβ.

For TGFz or Ki-67 staining, formaldehyde-fixed and de-waxed tissue sections were placed in a glass Coplin jar filled with 0.01 M FRG). Amino acids 144–160 of the C-terminus of GSTp (Biotrin International, Dublin, Eire); mouse monoclonal IgG against a synthetic peptide encompassing amino acids 144–160 of the C-terminus of proTGFβ (Ab-3; InnoGenex, San Ramon, CA, USA); mouse monoclonal antibody against full-length recombinant Ki-67 protein (Dianova, Hamburg, FRG).

For TGFz or Ki-67 staining, formaldehyde-fixed and de-waxed tissue sections were placed in a glass Coplin jar filled with 0.01 M sodium citrate buffer, pH 6.0. Slides were heated for periods of 2 min at a maximal power setting (about 800 W) and for 2 × 2 min at the submaximal power setting (600 W). The citrate buffer reached boiling point within 2 min and the fluid level in the Coplin jar was topped up with distilled water between heating periods to prevent drying of the sections. The following staining schedule was used: hydrogen peroxide to block endogenous peroxidases (3%, 20 min, room temperature); incubation with 2.5% bovine serum albumin (BSA) in TBS (0.05 M Tris, 0.3 M NaCl, pH 7.6; 30 min, room temperature); primary antibodies were diluted in 1% BSA-TBS (anti-Yp: 1:5000; anti-TGFβ: 1:50; anti-Ki-67: 1:50) and applied overnight at 4°C; rinsing with TBS; secondary antibodies were diluted in 2.5% BSA-TBS (biotinylated goat-anti-rabbit IgG or biotinylated rabbit-anti-mouse IgG; both 1:600, Dakopatts, Glosstrup, Denmark) and were applied for 90 min at room temperature; rinsing with TBS was followed by incubation with streptavidin–horseradish peroxidase conjugates (1:300 in TBS, 45 min, room temperature; Dakopatts); diaminobenzidine (Sigma, St Louis, MO, USA) was used for colour development. The specificity of immunohistochemistry was confirmed by omitting the primary antibodies.

Determination of DNA synthesis Rat liver 

\[^{3}H\)-thymidine (6.7 Ci mmol\(^{-1}\); NEN, Frankfurt, FRG) was injected into the peritoneal cavity as a single dose of 0.2 mCi kg\(^{-1}\) body weight) at the daily peak of DNA synthesis between 2000 and 2100 h (see above). After 36 h, animals were killed. GSTp-stained sections were coated with a solution of 1% gelatine (BioRad, Richmond, CA, USA) and 0.05% chromalaun (Merck, Darmstadt, FRG) and were air-dried. After autoradiography, the percentage of hepatocyte nuclei in DNA synthesis was determined for at least 1000 nuclei of unaltered cells in each liver and in all nucleated cells within individual GSTp\(^{+}\) foci (labelling index (LI)). Since interindividual variations were small, LIs obtained from different livers or foci of the same experimental group were pooled.

Human liver In order to identify human hepatocyte nuclei in the S-phase of the cell cycle, serial sections were stained for TGFz and Ki-67 (see above). Individual Ki-67-positive nuclei were followed in the consecutive TGFz-stained serial section by overlaying the two images in two microscopes linked by a bridge for over-projection (Zeiss, Germany).

Primary hepatocytes Male SPF Wistar rats were obtained from the animal facilities of the Medical University of Vienna at the age of 3–4 weeks and were treated with a single dose of NNM (250 mg kg\(^{-1}\) body weight), as described above. After 21 days, livers were perfused with collagenase as described (Parzefall et al, 1989; Löw-Baselli et al, 2000b). Treatment commenced 4 h after plating (time point 0). A stock of 10 μg (10 μl)\(^{-1}\) of 10 mM acetic acid of human recombinant mature TGFz (UBI, Lake Placid, NY, USA) was prepared and added to the medium for a final concentration of 10 ng ml\(^{-1}\). Tyrophostin A25 (synonym: tyrophostin AG82; Calbiochem, La Jolla, CA, USA) was dissolved in dimethylsulphoxide (DMSO) to obtain a stock of 10 mg ml\(^{-1}\); 1 μl of this stock was added per ml medium. Cypionate acetate, a gift from Schering AG (Berlin, FRG) was dissolved in DMSO. In all experiments, the final concentration of CPA was 10 μmol in 0.2% solvent. Recombinant mature TGF/f1 synthesised by CHO transfectants was supplied gratuitously by Bristol-Myers Squibb (Seattle, WA, USA). TGF/f1 was dissolved as described (Oberhammer et al, 1992).

Double immunostaining of culture plates for GSTp and TGFz - Cells in culture were fixed for 90 min at room temperature with 4% buffered formalin according to Lillic and were then kept in distilled water at 4°C until immunostaining. Then, the following schedule was used: hydrogen peroxide to block endogenous peroxidases (3%, 20 min, room temperature); primary antibodies were diluted in 2.5% BSA-TBS (biotinylated goat-anti-rabbit IgG or biotinylated rabbit-anti-mouse IgG, both 1:600, Dakopatts, Glosstrup, Denmark) and were applied for 90 min at room temperature; rinsing with TBS was followed by incubation with streptavidin–horseradish peroxidase conjugates (1:300 in TBS, 45 min, room temperature; Dakopatts); diaminobenzidine (Sigma, St Louis, MO, USA) was used for colour development. The specificity of immunohistochemistry was confirmed by omitting the primary antibodies.

Treatment of primary hepatocyte cultures Cells were seeded and kept under serum-free conditions, as described (Parzefall et al, 1989; Löw-Baselli et al, 2000b). Treatment commenced 4 h after plating (time point 0). A stock of 10 μg (10 μl)\(^{-1}\) of 10 mM acetic acid of human recombinant mature TGFz (UBI, Lake Placid, NY, USA) was prepared and added to the medium for a final concentration of 10 ng ml\(^{-1}\). Tyrophostin A25 (synonym: tyrophostin AG82; Calbiochem, La Jolla, CA, USA) was dissolved in dimethylsulphoxide (DMSO) to obtain a stock of 10 mg ml\(^{-1}\); 1 μl of this stock was added per ml medium. Cypionate acetate, a gift from Schering AG (Berlin, FRG) was dissolved in DMSO. In all experiments, the final concentration of CPA was 10 μmol in 0.2% solvent. Recombinant mature TGF/f1 synthesised by CHO transfectants was supplied gratuitously by Bristol-Myers Squibb (Seattle, WA, USA). TGF/f1 was dissolved as described (Oberhammer et al, 1992).

Determination of DNA replication in cultured hepatocytes Immunohistochemically stained plates were coated with 1% gelatine/0.05% hromalum, air-dried, dipped into photomulsion (Ilford K5, Dreieich, FRG), and exposed for about 14 h. The plates were processed with a photographical developer and fixative, and were finally dried at room temperature and mounted in Kayser’s glycerine gelatine (Merck, Darmstadt, FRG). The LI was calculated as percentage of labelled hepatocyte nuclei per total number of hepatocyte nuclei counted.

Two-dimensional gel electrophoresis, immunoblotting and identification of protein spots Nuclei and cytoplasm (postnuclear supernatant) of human hepatoma cells and of rat liver were separated according to the method of Tata, applying 2.0 M sucrose for purification (Tata, 1974). This was followed by a nuclear matrix preparation, as has been described in detail (Gerner et al, 1998).

The electropherocratically separated proteins were transferred onto PVDF sheets; the filters were soaked in excess blocking buffer (1% BSA, Sigma, St Louis, MO, USA) in TBST buffer (10 mM Tris–HCl, pH 8, 150 mM NaCl, 0.1% Tween 20)). The mouse monoclonal antisera against amino acids 39–88 of mature TGFβ (Oncogene Science, Ab-1, clone 134A-2B3; 1:300) were diluted in TBST buffer and were incubated overnight at 4°C. Thereafter, sheets were incubated for 1 h at room temperature with an alkaline phosphatase-conjugated anti-mouse IgG (Promega, Madison, WI, USA) diluted 1:7000 in TBST buffer containing...
0.25% BSA; 5-bromo-4-chloro-3-indolylphosphate and nitro blue tetrazolium chloride (Boehringer, Mannheim, FRG) were used for staining.

For mass spectrometry fingerprinting, Coomassie Blue-stained proteins were directly cut out of preparative gels. Matrix-assisted laser desorption ionisation-time-of-flight (maldi-tof) of tryptic protein hydrolysates and protein identification were carried out essentially as described (Fountoulakis and Langen, 1997; Grasl-Kraupp et al, 2002). Proteins were considered identified by means of mass spectrometry fingerprinting when at least 15% of the whole sequence gave hits and when the molecular mass/pl values were identical to the ones calculated or published in 2D databases.

Statistics

If not stated otherwise, data of at least three animals per time point and treatment group are given. Where indicated, the significance of differences of means was calculated by Kruskal–Wallis test or Wilcoxon test. For incidences, confidence intervals were calculated for $P<0.05$.

RESULTS

The pro-peptide of TGFz is present in the nuclei of human and rat hepatocytes and human hepatoma cell lines

In anti-TGFz-stained liver sections, immunoreaction was found within the cytoplasm, cell membranes and, most prominent, the nuclei of hepatocytes (Figure 1B, C and E). Likewise, the nuclei of the hepatoma cell lines HepG2 (Figure 1F), WRL68 and Hep2B (not shown) displayed a strong immunoreaction. Nuclear matrices, prepared from human HepG2 cells (Figure 2A) and from rat liver (Figure 2B), were separated by two-dimensional gel electrophoresis and were subsequently subjected to anti-TGFz immunoblotting; four spots around 17 kDa and an isoelectric point of 7.5 appeared. Spots 1–4 of HepG2 cells were subjected to maldi-tof analysis: spot 1 was verified to be the wild-type form of proTGFz; spot 2 was recently found to be wild-type proTGFz as well (Grasl-Kraupp et al, 2002). Spots 3 and 4 could not yet be identified. Immunostaining with antisera against the C-terminus of the pro-peptide also confirmed the presence of proTGFz in hepatocyte nuclei (see below). The mature form of TGFz at about 5.6 kDa was not detected.

Figure 1 Occurrence of nuclear proTGFz in human and rat hepatocarcinogenesis. (A–C) Rat liver 12 months post-NNM: serial sections of a preneoplastic lesion stained for GSTp (A) and TGFz (B); (C) HCC with proTGFz-pos nuclei (C). (D) Preneoplastic GSTp+ hepatocytes (violet) with proTGFz-pos nucleus (brown) and incorporated ^3H-thymidine (black spots) in primary culture; hepatocytes were isolated at day 21 post-NNM and cultured for 48 h; ^3H-thymidine was added to the medium 24 h before harvesting. (E) Human HCC and (F) human HepG2-hepatoma cells with proTGFz-pos nuclei. Magnifications: $\times 50$ for (A), (B), $\times 75$ for (E), $\times 25$ for (C); $\times 200$ for (D, F).
Occurrence of DNA replication and nuclear proTGFα in the different stages of human hepatocarcinogenesis

The percentage of both, replicating nuclei and proTGFα-pos nuclei, was gradually increased from normal liver, to hepatocellular adenoma, to dysplastic nodules, to HCC (Table 1, Figure 3). In unaltered liver, replicating nuclei and proTGFα-pos nuclei occurred at a similar frequency. Premalignant and malignant liver lesions, however, revealed a much higher percentage of proTGFα-pos nuclei when compared to nuclei in S-phase (Figure 1E and 3). Staining for Ki-67 was used to identify hepatocyte nuclei in the S-phase of the cell cycle (for details, see Materials and methods); five HCC were studied: 77.3 ± 2.9% of 343 Ki-67-positive hepatocyte nuclei evaluated displayed proTGFα. A high co-incidence of DNA synthesis and proTGFα was recently shown also for hepatocyte nuclei in cirrhotic liver (Grasl-Kraupp et al, 2002). Taken together, these findings indicate that a hepatocyte nucleus undergoing DNA replication almost always contains proTGFα. Further expression of proTGFα in nuclei not immediately undergoing DNA synthesis occurs predominantly in premalignant and malignant hepatocytes.

Occurrence of DNA replication and nuclear proTGFα in the different stages of rat hepatocarcinogenesis

Hepatocarcinogenesis was induced in rats by application of NNM. This leads to the formation of single GSTp⁺ cells and small preneoplastic GSTp⁺ lesions, followed by expansive growth of some of the lesions. To enhance the formation of liver tumours, rats were treated with the tumour promoter PB (Löw-Baselli et al, 2000b). Analogous to the findings in human liver, proTGFα-positive nuclei occurred in (pre)malignant hepatocytes at a significantly higher incidence than in unaltered hepatocytes (Figures 1A–C and 3). This substantiates that the experimental model fits the human situation.

Inherent growth advantage of cultured preneoplastic GSTp⁺ rat hepatocytes associated with nuclear proTGFα

To study the functional significance of nuclear proTGFα for hepatocarcinogenesis, unaltered GSTp⁻ and preneoplastic GSTp⁺ cells were isolated from the livers by collagenase perfusion and cultivated. Cell isolation was performed on day 21 post-NMN treatment (no PB promotion). At this time point, there is the maximal occurrence of GSTp⁺ cell clones in the liver. Based on stereological calculations of the size distribution in the third dimension, only 23 ± 11% of the isolated GSTp⁺ cells in culture derive from single-cell clones and 77 ± 29% derive from small foci (three cells on average), with an inherently elevated cell turnover (DeGust and Luebeck, 1998; Grasl-Kraupp et al, 2000).

Repetitive DNA synthesis was generally high in the isolated hepatocytes (Figure 4); this may be explained by the fact that the livers still underwent regeneration, as observed in vivo (Grasl-Kraupp et al, 2002). ProTGFα was present in the nuclei of about 10% of the hepatocytes in primary culture (Figure 4 and Table 2). In any case, DNA replication occurred preferentially in proTGFα-pos nuclei in both GSTp⁻ and GSTp⁺ hepatocytes (Table 2). This was also confirmed by immunostaining of parallel culture plates with two different antisera: DNA was synthesised by 76.5 ± 6.8% of the nuclei being positive for amino acids 39–88 of proTGFα (encompassing the mature form) and by 65.3 ± 19.2% of the nuclei being positive for amino acids 144–160 of the C-terminus of the pro-peptide. In both stains, DNA synthesis in negative nuclei was a rare event (see also Table 2).

GSTp⁺ cells showed an about three-fold higher frequency of proTGFα-pos nuclei and DNA replication than GSTp⁻ cells (Figure 4 and Table 2). Thus, the inherent growth advantage of the cultured preneoplastic cell population appears to be highly associated with the enhanced nuclear translocation of proTGFα.
Different signal transduction pathways of TGF\(\alpha\) gene products in GST\(p^+\) and GST\(p^-\) hepatocytes

We have confirmed recently that in our system mature TGF\(\alpha\) acts via erbB-1 in the cellular membrane of proTGF\(\alpha\)-neg cells according to classical concepts of growth signal transduction (Grasl-Kraupp et al., 2002). Accordingly, in the present study, addition of mature TGF\(\alpha\) increased DNA synthesis almost exclusively in the GST\(p^-\) cells that do not express proTGF\(\alpha\). This increase in DNA synthesis was blocked by the erbB-1-tyrosine kinase inhibitor tyrphostin A25 (Figure 5). However, mature TGF\(\alpha\) exerted no significant effect on any cell with nuclear proTGF\(\alpha\), which was most evident for the proTGF\(\alpha\)-rich GST\(p^+\) population (Figure 5). At present, it is unclear why TGF\(\alpha\) exerted no effect on the GST\(p^+\) cells with proTGF\(\alpha\)-neg nuclei. The expression of yet unidentified growth factors may confer autonomy from exogenous growth stimuli to this subpopulation of premalignant cells.

Taken together, these findings suggest that two different TGF\(\alpha\)-mediated signal transduction pathways are operative in different cell populations: the ‘classical’ erbB-1-mediated pathway of mature TGF\(\alpha\) becomes active mostly in the proTGF\(\alpha\)-poor GST\(p^-\) hepatocytes, while the second one, triggered by nuclear proTGF\(\alpha\), is effective in the proTGF\(\alpha\)-rich preneoplastic GST\(p^+\) cells.

DNA replication was suppressed by 1 ng of TGF\(\beta1\) and even more by 3 ng of TGF\(\beta1\) ml\(-1\) medium in GST\(p^-\) and GST\(p^+\) cells (Figure 6). DNA replication was inhibited at the most effective concentration of the cytokine in about 66% of the GST\(p^-\) cells, but in at least 90% of the GST\(p^+\) cells. These data suggest that TGF\(\beta1\) acts more strongly on GST\(p^+\) than on GST\(p^-\) cells. Furthermore, TGF\(\beta1\) diminished the fraction of hepatocytes expressing nuclear proTGF\(\alpha\) and synthesising DNA (Figure 6 and Table 2). Apparently, the suppression of DNA synthesis by TGF\(\beta1\) in primary hepatocytes involves downregulation of nuclear proTGF\(\alpha\). This effect was evident for both, GST\(p^-\) and GST\(p^+\) cells.

**Figure 4** DNA synthesis (LI(\%)) and \% of proTGF\(\alpha\)-pos nuclei in cultured GST\(p^-\) and GST\(p^+\) hepatocytes. \(^{3}\)H-thymidine was added 24 h before harvesting of cells. The \% of proTGF\(\alpha\)-pos nuclei was determined after 48 h of culture. Symbols: \(\Delta\) or open columns, GST\(p^-\) cells; ■ or dark columns, GST\(p^+\) cells. In each of the experiments, 2000 nuclei of GST\(p^-\) cells and 600 nuclei of GST\(p^+\) cells were evaluated. Means \(\pm\) s.d. are given from separate experiments with cultures from five rats. Statistics of \% of proTGF\(\alpha\)-pos nuclei in GST\(p^-\) vs GST\(p^+\) cells over time-course by Kruskal--Wallis test: (a) \(P<0.001\). Statistics of LI(\%) in GST\(p^-\) cells vs GST\(p^+\) cells at the last time point of evaluation by Wilcoxon’s test: (b) \(P<0.01\). Statistics of \% of proTGF\(\alpha\)-pos nuclei in GST\(p^-\) vs GST\(p^+\) cells by Wilcoxon’s test: (c) \(P<0.001\).

**Figure 5** Mature TGF\(\alpha\) increases the percentage of nuclei in S-phase (LI(\%)) preferentially in GST\(p^-\) hepatocytes without nuclear proTGF\(\alpha\). \(^{3}\)H-thymidine was added 24 h before harvesting of cells. The percentages of replicating and of proTGF\(\alpha\)-pos nuclei were determined after 48 h of culture. For the number of experiments and cells scored, see Figure 4. Hatched portions of the bars indicate LI of proTGF\(\alpha\)-pos nuclei; nonhatched portions of the bars indicate LI of proTGF\(\alpha\)-neg nuclei; the sum of the hatched and unhatched portion (total bar) indicates LI of all nuclei. Co: DMSO-control; TGF\(\alpha\): mature TGF\(\alpha\); Tyr: Tyrophostin A25. Statistics by Student’s \(t\)-test: (a) \(P<0.05\).

**Table 2** DNA synthesis occurs preferentially in proTGF\(\alpha\)-pos nuclei

|                     | CO                  | CPA                  | TGF\(\beta1\) (10 ng) |
|---------------------|---------------------|----------------------|-----------------------|
|                     | In GST\(p^-\)      | In GST\(p^+\)       | In GST\(p^-\)         | In GST\(p^+\)       |
| % of proTGF\(\alpha\)-pos nuclei | 10.3 \(\pm\) 2.9 | 29.0 \(\pm\) 5.9*   | 20.7 \(\pm\) 3.2**    | 46.1 \(\pm\) 7.2*   |
| % of proTGF\(\alpha\)-pos nuclei in S phase | 76.3 \(\pm\) 6.6 | 62.1 \(\pm\) 7.8     | 81.7 \(\pm\) 4.3      | 75.1 \(\pm\) 5.8    |
| % of proTGF\(\alpha\)-neg nuclei     | 89.3 \(\pm\) 2.9  | 71.0 \(\pm\) 5.9*** | 79.3 \(\pm\) 3.2      | 53.9 \(\pm\) 7.2****|
| % of proTGF\(\alpha\)-neg nuclei in S phase | 4.3 \(\pm\) 1.3  | 6.9 \(\pm\) 4.9      | 9.6 \(\pm\) 3.6       | 12.5 \(\pm\) 5.7    |

\(^{3}\)H-thymidine was added 24 h before harvesting of cells. The percentage of nuclei in S phase and of proTGF\(\alpha\)-pos nuclei were determined after 48 h of culture. In each experiment, 2000 nuclei of GST\(p^-\) cells and 600 nuclei of GST\(p^+\) cells were evaluated. Means \(\pm\) s.d. are given from separate experiments with cultures from five rats. Statistics by Wilcoxon’s test for GST\(p^-\) cells vs GST\(p^+\) cells. \(*\ast\ast<0.05\), \(*\ast\ast<0.01\), \(*\ast<0.001\); for CO vs treated groups: \(*\ast\ast\ast<0.005\), \(*\ast\ast\ast<0.01\).
followed by replicative DNA synthesis (Grasl-Kraupp et al., 2002). Nuclear translocation of proTGFz is induced by various hepatomitogenic stimuli, such as regenerative growth after partial hepatectomy or intoxication with CCl₄ and hyperplastic growth induced by CPA in the intact animal. In culture, the incidence of proTGFz-pos hepatocyte nuclei is elevated by treatment with hepatomitogenic CPA, prostaglandins E₂ and F₂α, and hepatocyte growth factor (Grasl-Kraupp et al., 2002; Schausberger et al., 2003). Thus, a great variety of growth stimuli all involve nuclear translocation of proTGFz. This peptide may therefore serve as a kind of intracellular shortcut in mediating autocrine growth stimulation of normal, premalignant and malignant liver cells.

The possible interactions of proTGFz with the growth-regulatory machinery in the nucleus of unaltered and (pre)neoplastic hepatocytes are still unclear. Soluble or membrane-bound precursors of TGFz are biologically active, suggesting that the pro-form may attach to the binding site of erbb-1 as known for the mature form (Ignotz et al., 1986). We found that proTGFz and erbb-1 almost always co-localise within the nucleus of mouse hepatocytes, as shown by confocal laser-scanning microscopy (Schausberger et al., 2003). It is currently under investigation by FRET technology whether the large TGFz precursor attaches to erbb-1 and may be co-targeted to the nucleus as a receptor-bound ligand. This may provide the clue for the function of this pro-peptide for DNA synthesis, considering that erbb-1 may act as a transcription factor for cyclin D₁ (Lin et al., 2001). Our data also suggest that the activity of proTGFz in the nucleus does not depend on an erbb-1 receptor tyrosine kinase activity, since DNA replication of proTGFz-pos nuclei was not affected by the tyrosine kinase inhibitor tyrphostin, after DNA replication of proTGFz-pos nuclei was not affected by the tyrosine kinase inhibitor tyrphostin (Figure 5).

The cell culture system used in the present study allows to investigate the functional significance of nuclear proTGFz in premalignant cells; cultured GSTp⁺ hepatocytes showed significantly higher basal rates of DNA replication than GSTp⁻ hepatocytes. These characteristics closely reflect those described for GSTp⁻ cells in the intact liver in vivo. Thus, this defect in growth regulation persists under culture conditions and therefore appears to be independent of intercellular contacts within the intact organ, and of cytokines, growth factors or hormones circulating in the whole body. The present study shows that considerably more GSTp⁺ cells synthesise and transport proTGFz to the nucleus than GSTp⁻ cells. The nuclear import of this pro-peptide almost always is followed by DNA replication. The enhanced probability of nuclear translocation of proTGFz may therefore be essential for the intrinsic growth advantage of the preneoplastic cell population. Compared to the current concepts of signal transduction, nuclear proTGFz may not depend on intact erbb-1 receptors on the cell surface. It circumvents the secretion and possible loss of TGFz to the outside of the cell and may confer autonomy and an inherent growth advantage, a pathway preferentially used by the preneoplastic cell population.

In an untreated, healthy liver, almost all of the hepatocytes are in the G₀-phase of the cell cycle. In the present study, the incidence of nuclei positive for proTGFz increased in the course of hepatocarcinogenesis. Since proTGFz translocates to the nucleus in the G₁-phase of the cell cycle (Grasl-Kraupp et al., 2002), the elevated presence of nuclear proTGFz in (pre)malignant hepatocytes may be evidence for a G₁-status of these cells. Several groups reported that premalignant liver cells show an increased expression of c-myc and cyclin D₁, known inducers of the transition from G₀ to G₁ of the cell cycle (Deguchi and Pitot, 1995; Ramlijak et al., 2000). On the other hand, the expression of the WAFI/CIP1 gene product, p21 and the c-myc antagonist mad were decreased in hepatocarcinogenesis (Martens et al., 1996). Many growth-stimulating factors exert their activity, provided that the target cell is in the G₁-phase of the cell cycle. Thus, the enhanced presence of nuclear proTGFz in the (pre)neoplastic cell compartment appears to be involved in...
the overcoming of critical checkpoints of the cell cycle and in a facilitated response of liver (pre)neoplasia towards various growth stimuli.

Transforming growth factor \( \alpha \) and erbB-1 are upregulated in malignancies of many different organs, including HCC. Novel therapeutic approaches have been focusing on the possible benefit of blocking TGF\( \alpha \)-evoked signal transduction on the cell surface, for example, by erbB-1 blockade (Levitzki and Gazit, 1995; Mendelsohn, 1997). The present study shows that treatment with mature TGF\( \alpha \) stimulated DNA synthesis rather in proTGF\( \alpha \)-neg than in proTGF\( \alpha \)-pos hepatocytes, which was abrogated by an erbB-1-specific tyrosine kinase inhibitor. In GSTP \( \alpha \) hepatocytes, however, mature TGF\( \alpha \) and tyrphostin exerted no significant effect. Thus, the ‘classical’ signal transduction pathway of mature TGF\( \alpha \) via erbB-1 seems to be active in the unaltered cell population, while the novel pathway seems to operate preferentially in the (pre)malignant cell compartment. It is therefore tempting to speculate that liver tumours may use alternative pathways for growth stimulation by proTGF\( \alpha \). Then, they may be resistant against therapeutic strategies targeted at TGF\( \alpha \)/erbB-1 interactions on the cell surface.

In conclusion, the present work shows that the incidence of nuclei expressing proTGF\( \alpha \) is elevated in the course of rat hepatocarcinogenesis, which may reflect and contribute to the inherent growth advantage of (pre)neoplastic hepatocytes. Further research is necessary to elucidate the mechanisms that regulate the different intracellular routes of proTGF\( \alpha \) and that may link nuclear proTGF\( \alpha \) to DNA replication.

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