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Liver Tyrosine Kinase Activation During Early Stages of Chemical Hepatocarcinogenesis

Jack W. Olson

Division of Pharmacology and Toxicology, University of Kentucky College of Pharmacy, Lexington, Kentucky 40536

Protein phosphorylation at tyrosine residues is believed to be involved in several important cellular processes because tyrosine-specific protein kinase activation is associated with stimulation of cellular proliferation by hormones and growth factors, embryogenesis, and retroviral cell transformation. Because cell proliferation is thought to be an essential component of chemical carcinogenesis, liver tyrosine-specific protein kinase activity was examined during the early stages of the Solt and Farber chemical hepatocarcinogenesis model. Rats were given diethylnitrosamine in one dose (200 mg/kg, IP) followed by 2 weeks of dietary 0.02% 2-acetylamino-fluorene starting at day 14 after diethylnitrosamine, followed by partial hepatectomy on day 21. By day 32 this regimen produces a relatively synchronized population of hyperplastic liver nodules up to 1.5 mm in diameter. Rats were sacrificed on day 32, their livers were perfused with cold normal saline, homogenized, and centrifuged at 1,000g for 10 min. The resulting supernatant was centrifuged at 30,000g for 30 min and the pellet was assayed for tyrosine kinase activity using the synthetic peptide [Val^5]angiotensin II as substrate. Rats that received the complete regimen had a 2.6-fold increase in their liver tyrosine kinase activity as compared to sham controls (2.4 pmoles/min/mg protein vs 6.4 pmoles/min/mg protein, $P < .05$). In contrast, rats that received a partial regimen (ie, partial hepatectomy, or 2-acetylaminofluorene + partial hepatectomy, or diethylnitrosamine + 2-acetylaminofluorene) did not have elevated tyrosine kinase activity nor did they have hyperplastic nodules. These preliminary data suggest that activation of liver tyrosine kinase is associated with the very early stages of chemical hepatocarcinogenesis.

Key words: tyrosine kinase, hepatocarcinogenesis, hyperplastic nodules

Tyrosine protein kinase activity and/or specific phosphorylation of protein tyrosine residues has been shown to be associated with normal and malignant cellular proliferation. For example, tyrosine-specific protein kinase activity appears to be an intrinsic property of a number of RNA viral transforming gene products and their cellular homologs [reviewed in 1,2]. Membrane receptors for a variety of growth

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factors, such as epidermal growth factor [3,4], platelet-derived growth factor [5], and insulin [6], are associated with tyrosine kinase activity. In addition, growth factor stimulation of cellular proliferation is also associated with an increase in tyrosine kinase activity [2]. The relatively recent use of tyrosine containing peptide substrates for in vitro assay has demonstrated that tyrosine protein kinases are found in both normal proliferating and nonproliferating cells [7–11]. Proliferating tissue such as cultured human placenta has high tyrosine kinase activity [11] and tyrosine kinase activity increases during early embryonic development of the sea urchin [10], whereas the liver, a normally nonproliferating tissue, has low tyrosine kinase activity [7,9].

Evidence has accumulated that strongly suggests that both initiation and promotion stages of chemical carcinogenesis have a requirement for cell proliferation [reviewed in 12,13]. In the Solt and Farber multistage chemical hepatocarcinogenesis model [14], regenerative hyperplasia is produced during initiation by a single dose of the carcinogen diethylnitrosamine (DEN) and by partial hepatectomy during the promotionlike stage. This model offers the advantage of rapidly producing relatively synchronized early hyperplastic nodules whose development can be monitored and quantitated by the putative preneoplastic enzyme marker γ-glutamyltranspeptidase (GGT) [12–14]. This work tests the hypothesis that liver tyrosine kinase activity is increased during the early proliferative stages of chemical hepatocarcinogenesis. The reported data demonstrate that liver tyrosine kinase activity is selectively increased only by a complete carcinogenic treatment regimen that produces GGT-positive hyperplastic nodules.

MATERIALS AND METHODS

Animals and Carcinogenic Treatment

Male Fischer 344 rats (150–170 gm) were maintained on a standard high-protein (24%) basal diet (Dyets, Inc., Bethlehem, PA) and water ad libitum for 7 days prior to initiation of experiments. The rats were housed in hanging wire cages in a room kept at constant temperature with a 6 AM to 6 PM photoperiod.

Animals were divided randomly into several control and treatment groups. The complete carcinogenic treatment regimen was similar to that of Solt and Farber [14] and is shown in Figure 1. Briefly, 2 weeks after a single dose of DEN (200 mg/kg, IP), rats were placed on the basal diet containing 0.02% 2-acetylaminofluorene (2-AAF) (Dyets, Inc.) for 14 days. One week after initiation of the 2-AAF diet, a standard two-thirds partial hepatectomy (PH) was performed. After receiving 14 days of the diet containing 2-AAF, the animals were returned to the standard basal diet. Additional groups of rats served as controls (saline injection + sham hepatectomy [SH]) or received the following partial treatment regimen: saline injection + PH only, or saline injection + 2-AAF diet + PH, or DEN + 2-AAF diet + SH.

Preparation of Liver Extracts

All animals were sacrificed by cervical dislocation between 9 AM and 11 AM; their livers were immediately perfused with 40 ml of cold 0.9% NaCl and the caudate liver lobe was rapidly removed and frozen in liquid nitrogen. Frozen liver samples were homogenized using an Ultra-Turrax homogenizer at low speed for 30 sec in 5 volumes of cold buffer A (50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride) and followed by four bursts for 10 sec

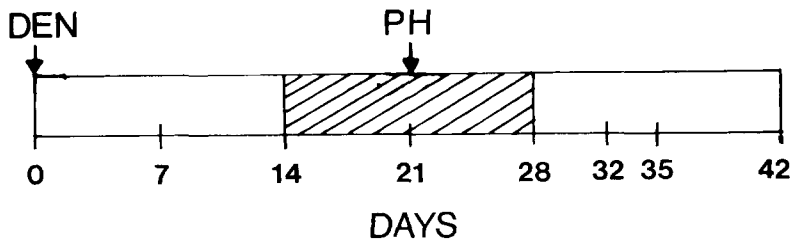

Fig. 1. Schematic representation of complete carcinogenic treatment regimen. Rats were given diethylnitrosamine (DEN) in one dose (200 mg/kg, IP) followed by 2 weeks of dietary 0.02% 2-acetylaminofluorene (cross-hatched area) starting at day 14 after DEN, followed by partial hepatectomy (PH) on day 21. All rats were sacrificed on day 32.

each at high speed (setting 10). The homogenates were centrifuged at 1,000g for 10 min and the resulting supernatant at 30,000g for 30 min [9].

Kinase Assay

The 30,000g particulate fraction was suspended in buffer A and assayed for tyrosine kinase activity utilizing a method similar to Wong and Goldberg [7] and Swarup et al [9] with the synthetic peptide, [Val^5]angiotensin II as substrate [7,15] in a total reaction volume of 25 μl. The final incubation mixture contained 50 mM Tris-HCl, pH 7.8, 25 mM Mg acetate, 10 μM Na vanadate, 0.05% Nonidet P-40, 60 μM [γ-^{32}P]ATP, 13–17 μg of the particulate supernatant and plus or minus 25 μg of peptide. The assay was initiated by the addition of ATP, incubated for 10 min at 30°C, and stopped by the addition of 75 μl of 3.3% trichloroacetic acid, followed by 10 μl of bovine serum albumin (20 mg/ml). After centrifugation at 1,500g for 30 min, 90 μl of the resulting supernatant fraction was spotted on phosphocellulose paper (Whatman P-81), and the papers were washed five times in 0.5% phosphoric acid and once in acetone. The filter papers were counted in 10 ml of Toluene/Omnifluor scintillant. Utilizing the above assay conditions, the amount of ^{32}P incorporated into the peptide was linear with respect to both time and kinase concentration. The protein kinase activity reported is the difference between the appropriate samples incubated with or without the peptide. The amino acids phosphorylated in the kinase reaction supernatant fraction were determined by acid hydrolysis and subsequent thin layer electrophoresis at pH 3.5 as described by Hunter [16].

γ-Glutamyltranspeptidase Histochemistry

The right anterior and posterior lobes of perfused liver were fixed in cold 5% acetic acid and embedded in paraffin and GGT histochemistry was performed on 4-μm tissue sections according to the method of Rutenberg et al [17] as modified by Ogawa et al [18]. The total number of GGT-positive hyperplastic nodules per cm^2 of liver section was determined.

Materials

Diethylnitrosamine (DEN) was obtained from Eastman Kodak Company (Rochester, NY), [γ-^{32}P]ATP (10 Ci/mmol) from New England Nuclear (Boston, MA), phosphocellulose P-81 paper, Whatman. All other reagents were obtained from Sigma (St. Louis, MO), Fisher (Pittsburgh, PA), or Pierce Chemical Co. (Rockford, IL).

TABLE I. Number of GGT-Positive Hyperplastic Nodules

Treatment[a]	No. of rats	Total No. of nodules/cm^2 liver section
Saline + SH	4	0[b]
Saline + PH	4	0
Saline + 2-AAF + PH	4	0
DEN + 2-AAF + SH	4	0
DEN + 2-AAF + PH	4	60 ± 16

[a]Rats were subjected to partial treatment regimes or the complete regimen as described in Figure 1. All rats were sacrificed 32 days after receiving either a single injection of saline or DEN. SH, sham hepatectomy.
[b]No GGT-positive nodules were detectable.

RESULTS

GGT-positive hyperplastic liver nodules were produced by the complete carcinogenic treatment regimen (Table I). These hyperplastic liver nodules varied in size but were as large as 1.5 mm in diameter at day 32. It is important to note that rats that received a partial treatment regimen did not develop GGT-positive liver nodules. These results are essentially the same as previously reported by Solt and Farber [14].

Compared to livers from control rats, tyrosine protein kinase activity was significantly increased 2.6-fold in livers from rats exposed to the complete regimen (Table II). This is in contrast to the effect of partial treatment regimens, which did not alter liver tyrosine kinase activity with respect to the saline + sham controls (Table II). In addition, only the complete treatment regimen caused an increase in background tyrosine kinase activity, that is, activity assayed in the absence of exogenous peptide (data not shown). Thin-layer electrophoresis of the acid hydrolyzed supernatant fluid from the kinase assay mixture demonstrated that tyrosine was the only amino acid phosphorylated (data not shown).

DISCUSSION

The results of the present study clearly show that liver tyrosine protein kinase activity is significantly increased following chemical carcinogenic treatment. This increase in tyrosine protein kinase activity coincided with the development of grossly visible focal collections of proliferating hepatocytes (ie, hyperplastic nodules). It is significant that complete or partial treatment regimens did not alter liver tyrosine protein kinase activity as compared to controls nor did these regimens produce GGT-positive hyperplastic nodules. However, assays were performed on subcellular fractions obtained from intact liver lobes comprised of several cell types in addition to GGT-positive cells. Thus the observed increase in tyrosine kinase activity may not be derived entirely from GGT-positive cells.

The present experiment compared rat liver tyrosine kinase activity following treatment with a complete carcinogenic regimen that produces liver cells still replicating by day 32 versus partial treatment regimens that result in liver cells no longer rapidly proliferating by day 32 [14,19]. Therefore, it remains to be demonstrated

TABLE II. Liver Tyrosine Protein Kinase Activity

Treatment[a]	No. of rats	Kinase activity[b] (pmol/min/mg protein)
Saline + SH	4	2.4 ± .12
Saline + PH	4	2.3 ± .40
Saline + 2-AAF + PH	4	2.6 ± .06
DEN + 2-AAF + SH	4	2.1 ± .18
DEN + 2-AAF + PH	4	6.4 ± .48[c]

[a]Rats were subjected to partial treatment regimens or the complete regimen as described in Figure 1. All rats were sacrificed 32 days after receiving either a single injection of saline or DEN. SH, sham hepatectomy.
[b]Mean ± S.E. of triplicate determinations on each rat liver. The kinase activity reported is the difference between samples incubated with peptide minus samples incubated without peptide.
[c]Data differe from saline + SH controls at $P < 0.05$.

whether the activation of tyrosine kinase(s), as estimated by exogenous peptide phosphorylation, is unique to this complete carcinogenic regimen or if it also occurs during noncarcinogenic liver cell replication. Although the Solt and Farber model produces a relatively synchronous group of rapidly proliferating nodules [19], several other hepatocarcinogenesis models utilize treatment regimens that produce foci that develop very slowly into nodules [reviewed in 20]. It would be important to determine whether or not activation of liver tyrosine kinase(s) occurs in these slowly developing nodules produced by different models of hepatocarcinogenesis. The majority of the nodules produced by the various hepatocarcinogenesis models will "regress" or "remodel" [12,13]; however, a few nodules will eventually become a site of origin for hepatocellular carcinomas [12–14]. The data presented here demonstrate that increased liver tyrosine protein kinase activity occurs at a very early stage of chemical hepatocarcinogenesis. It has yet to be determined whether or not this increase in enzyme activity is specifically associated with those persistent nodules that undergo progression to malignancy.

Transforming retroviral oncogene products with tyrosine kinase activity have been observed in a number of malignant tumors, but it is not known at what stage of chemical carcinogenesis these specific oncogenes are activated [1,2]. Although the present study demonstrates that the very early stages of hepatocarcinogenesis are associated with increased liver tyrosine kinase activity, it does not address whether or not this increased enzyme activity is a function of an oncogene product. However, it may be relevant that several studies have recently demonstrated that normal nonproliferating liver has low levels of tyrosine kinase activity [7,9]. In addition, epidermal growth factor has been reported to stimulate tyrosine phosphorylation of a protein from liver microsomal fractions and plasma membranes [21]. Furthermore, it was reported that phosphorylation of liver membrane tyrosine residues was altered 36 hr after a partial hepatectomy [21]. Characterization of the carcinogen-activated liver tyrosine protein kinase reported in this paper is in progress.

In conclusion, these results indicate that there is selective regulation of liver tyrosine protein kinase activity during the early stages of chemical hepatocarcinogenesis. The significance of this observation remains to be determined.

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