Evaluation of tumour necrosis factor-α, soluble P-selectin, γ-glutamyl transferase, glutathione S-transferase-π and α-fetoprotein in patients with hepatocellular carcinoma before and during chemotherapy

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Accepted: 3 September 2005

ABSTRACT

Hepatocellular carcinoma (HCC) is an environmentally related cancer, with both viral and chemical carcinogens involved in a multistage process. To date, it has been difficult to detect the asymptomatic precursor lesions in early HCC. Therefore, the majority of HCC patients are not amenable to therapy, as they are detected at late stages. To evaluate the significance of tumour necrosis factor-α (TNF-α), sP-selectin, γ-glutamyl transferase (GGT), glutathione S-transferase-π (GST) and α-fetoprotein (AFP) in the diagnosis and follow up of HCC patients during chemotherapy with adriamycin, 45 subjects (15 healthy volunteers, 15 with benign liver diseases and 15 HCC patients) are studied before and during chemotherapy (three cycles of intravenous adriamycin). HCC patients had significantly higher serum levels of TNF-α, sP-selectin, GGT, GST and AFP. Serum levels of GGT and GST were significantly higher in HCC patients with poorly differentiated tumours than in patients with well- and moderately differentiated tumours. Treatment with adriamycin for three cycles produced a significant decrease in TNF-α, sP-selectin and GST. Thus, it is concluded that GST is a superior diagnostic indicator and may be a prognostic marker in HCC patients.

KEY WORDS: Hepatitis B virus.
Hepatitis C virus.
Hepatocellular carcinoma.
Neoplasms, liver.

Introduction

Hepatocellular carcinoma (HCC) is a malignancy that shows high mortality, with one million new cases seen worldwide each year. It has a male:female ratio of 4:1. While HCC is relatively uncommon in Western countries, it is the most common cancer in African and Far East populations due to hyperendemic hepatitis in these areas.[1,2] In Egypt, HCC represents 7% of all cancer cases and shows a male:female ratio of 3:1.

Chronic hepatitis B and C virus (HBV and HCV) infections, with and without schistosomiasis, dietary aflatoxin B (AFB) and chemical carcinogen exposure, cigarette smoking and low consumption of vegetables are the main risk factors associated with HCC development.[1,2]

The contribution of cytokines to the development and progression of HCC is well documented.[2] Cytokine production is thought to play a role in regulating the recruitment of tumour- associated inflammatory cells,[3] in the induction of angiogenesis[4] and in the direct modulation of tumour cell proliferation.[5]

Tumour necrosis factor-α (TNF-α) is a 17 kDa protein produced primarily by mononuclear phagocytes and lymphocytes.[6] It has been reported that TNF-α is involved in the pathogenesis of diverse liver diseases including viral hepatitis and HCC.[7] The immunomodulatory effect of TNF-α is achieved via binding to specific cellular receptors (TNF-Rs), TNF-R55 and TNF-R75.[8] The TNF–TNFR system has been implicated in a wide range of biological functions including cytotoxicity against tumours and virus-infected cells, direct antiviral and pro-inflammatory activities, as well as stimulation of many immune effector cells.[9] TNF-α plays a central role in up-regulating cell surface adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin, thereby promoting adhesion of leucocytes to and subsequent transmigration through the endothelial barrier.[10]

Expression of adhesion molecules, which leads to the interaction of particular sets of cells, may be the primary signal for the activation and extravasation of immune cells to the site of inflammation.[11] P-selectin (CD62, GMP-140,

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PADGEM) belongs to the selectin family of adhesion molecules. P-selectin acts as a receptor that supports binding of leucocytes to activated platelets and endothelium. P-selectin-mediated adhesive interaction operates in conjunction with cell-cell interactions directed by related molecules, and is likely to be important in both haemostatic and inflammatory processes.[12] The physiological role of P-selectin might be the mediation of initial leucocyte adhesion to activated endothelium during acute inflammation. It may work in concert with E-selectin to direct early, regionally specific adherence of neutrophils and monocytes at sites of acute inflammation.

A soluble form of P-selectin, which might represent a proteolytic fragment or more likely a soluble splice variant lacking the transmembrane domain, has been detected in serum and plasma in cancer patients.[13] P-selectin has an important role in tumour formation and metastasis, as malignant cells are shown to express receptors for P-selectin.[14] Soluble adhesion molecules (sP-selectin), like their membrane-bound counterparts, may affect cell-cell interactions. Thus, identification of soluble forms of these molecules provides the potential for further clinical study of their role in the monitoring of inflammatory and malignant diseases.[15]

γ-glutamyltransferase (GGT) is a membrane-bound ectoenzyme that catalyses the degradation of glutathione and other γ-glutamyl compounds.[16] Serum GGT activity is a sensitive marker of hepatobiliary disorders. It is generally accepted to be the most sensitive marker of cholestasis and pancreatic disease or enzymatic induction by alcohol and drugs.[17] Serum GGT levels are affected by several other factors and a variety of clinical conditions (i.e., measurement of total serum GGT lacks specificity) and its value in the differential diagnosis is limited due to overlap in patients with benign and malignant liver diseases.[18] γ-glutamyltransferase mediates the uptake of glutathione by breaking down extracellular glutathione, making its amino acid component available to the cell.[19]

Glutathione S-transferase-π (GST) is an acidic 25 kDa dimeric protein. It is a detoxifying isoenzyme that catalyses the nucleophilic addition of glutathione at electrophilic centres in a wide variety of compounds, making removal of such xenobiotics from the body possible. The amount of GST in primary hepatomas is more than 10-fold higher than in the normal liver.[1, 20]

α-fetoprotein (AFP) is a well-established cell differentiation and tumour marker. Activity is high in fetal and post-natal hepatocytes but is almost undetectable in adult liver parenchymal cells. However, AFP production can resume in the liver under pathological conditions such as primary HCC. Elevated AFP level is regarded as a sensitive marker for the diagnosis of HCC. Despite the fact that false-positive and false-negative serum AFP results are seen (e.g., in schistosomal hepatic fibrosis [SHF], where 20–30% patients with HCC had a negative AFP result, while some cases of chronic hepatitis and cirrhosis showed a positive result), it is an important diagnostic test in HCC.[22]

The aim of the present study is to determine the levels of TNF-α, sP-selectin, GGT, GST and AFP in patients with HCC before and after three cycles of a single intravenous chemotherapeutic agent (adriamycin) to validate their value as tumour markers in HCC diagnosis and follow up.

Materials and methods

This study comprised 45 subjects divided into three groups of matched age and gender. In the benign group there were 15 farmers with SHF (12 males, three females). The age range was 51–64 years. In the past they had received treatment (PRQ, 40–60 mg/kg) as a single dose but none were receiving treatment at the time of the study.

In the malignant group there were 15 patients with HCC, selected from the hospital and the oncology clinic of the Medical Research Institute, Alexandria University, between April 2001 and October 2002. Patients had received three cycles of a single chemotherapeutic agent (50 mg adriamycin/m^2 of body surface area) given intravenously every three weeks.[23]

The control group comprised 15 normal healthy volunteers.

Clinical investigations and collection of samples

All groups were subjected to HBV and HCV serological tests. Patients with HCC were subjected to a thorough clinical examination and routine laboratory investigations, including complete blood count (CBC) and liver function tests, ultrasonography (u/s) of the liver, chest X–ray (when needed) and u/s-guided needle biopsy of the liver to establish the pathological diagnosis.

Serum samples from all three groups were used for determination of TNF-α, sP-selectin, GGT, GST and AFP.

Determination of serum TNF-a and sP-selectin

Determination of serum TNF-α and sP-selectin was carried out using a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA) kit (ImmunoTech, France, for TNF-α and Bender MedSystems for P-selectin). Briefly, serum samples were added to the wells of microtitre plates precoated with specific monoclonal antibodies to TNF-α and sP-selectin. After incubation at room temperature and washing of unbound antigen, the enzyme-linked polyclonal antibodies specific for TNF-α and P-selectin were added to the wells. After washing to remove unbound enzyme, the substrate was added to induce a coloured reaction product. The colour reaction was stopped and the intensity was measured at 450 nm. Intensity of the coloured produced was directly proportional to the concentration of TNF-α or P-selectin present in samples. Results were expressed as pg/mL for TNF-α and ng/mL for sP-selectin.

Determination of serum GGT

Serum GGT was determined by the method of Rosalki and Tarlow,[24] in which the almost colourless L-γ-glutamyl-p-nitroaniline was used as substrate. The enzyme γ-glutamyl transferase liberates yellow p-nitroaniline so that an increase in colour provides a measure of enzyme activity. Glycylglycine was used as the glutamyl acceptor.

Preparation of ^{125}I-labelled GST and measurement of serum levels

Glutathione S-transferase-π was labelled with iodine 125 using the chloramine–T reaction by a modification of the method of Hayes *et al*.[25] In brief, GST (Sigma, 5 μg) and ^{125}I-labelled sodium iodide (MDS, Belgium, 500 μCi [carrier free]) were added to a Reactivial (Pierce, USA), followed by chloramine–T (16 μg). After 30 sec the reaction was stopped by the addition of cysteine (56 μg), potassium iodide (100 μg)

Table 1. Results of serum TNF-α, sP-selectin, GGT, GST and AFP in the control, benign (SHF) and malignant (HCC) groups studied.

	Controls	SHF group	Malignant (HCC)	
			Before chemotherapy	After chemotherapy
TNF-α (pg/mL)				
Min – Max	6.4–17.8	175–321	256–499	200–361
Mean ± SE	12.4 ± 0.98	248.3 ± 12.1*	270.0 ± 19.3*†	258.7 ± 18.4*‡
sP-selectin (ng/mL)				
Min – Max	149.0–264.0	190.0–285.0	260.0 – 350.0	210–290
Mean ± SE	204.4 ± 9.9	243.5 ± 8.5*	306.1 ± 7.2*†	251.4 ± 7.6*‡
GGT (U/L)				
Min – Max	4–12	29–48	79–1229	50–392
Mean ± SE	8.6 ± 0.7	39.0 ± 1.2*	253.6 ± 81.8*†	168.7 ± 34.1*
GST (mg/L)				
Min – Max	0–35	0–48	33–155	18.5–87
Mean ± SE	14.0 ± 3.2	20.7 ± 4.9*	62.7 ± 6.3*†	43.2 ± 5.5*‡
AFP (iu/mL)				
Min – Max	0–1.4	1.1–10.0	2.5– 400	0–480
Mean ± SE	0.91 ± 0.22	3.2 ± 0.69*	43.3 ± 15.5*†	30.0 ± 7.3*

SE: standard error; *significant when compared with controls; †significant when compared with benign; ‡significant when compared with before chemotherapy.

and elution buffer (250 µL). The contents of the Reactivial were then equilibrated with the elution buffer and transferred to a gel filtration chromatographic column (1.6 x 35 cm) packed with Sephadex G-25 (fine). The labelling reaction mixture was applied to the column and was eluted with the elution buffer. Fractions with the highest radioactivity (CPM) and protein content (A_{280}, Lowry method)[26] were used as the tracer in an improved radioimmuonassay (RIA) kit. Serum GST levels in the different studied groups were measured using a modified RIA procedure[27] in which the first antibody was rabbit anti-human GST (Dako, diluted 1 in 25) and the second antibody was swine anti-rabbit immunoglobulin (Dako, diluted 1 in 10). Results were expressed as µg/L.

Determination of serum AFP

Serum AFP was measured using a ready-to-use immunoradiometric assay (IRMA) kit (Diagnostic Products, USA).[1] Results were expressed as iu/mL.

Statistical analysis

Data were subjected to analysis of variance using the general model procedure (SAS Institute, 1994). Variables with a significant *P* test ($P<0.05$) were compared using the least significant difference (LSD) test.[28]

Results

No viral hepatitis was detected in the control group. HBV and/or HCV were detected only in the SHF and HCC groups, with rates of 40% (6/15) and 67% (10/15), respectively.

Table 1 shows the significant elevation in serum levels of TNF-α, sP-selectin, GGT, GST and AFP in the malignant (HCC) group prior to chemotherapy, compared to results for the benign (SHF) and control groups.

After three cycles of a single intravenous chemotherapeutic agent (adriamycin), the malignant group showed a significant decrease in serum levels of TNF-α, sP-selectin and GST. No correlation was observed between the studied parameters and tumour size in the HCC group (Table 2).

In the HCC group (Table 3), serum levels of GGT and GST were significantly higher in HCC patients with poorly differentiated tumours than in patients with either well-differentiated or moderately differentiated tumours. Levels of TNF-α, sP-selectin and AFP were not significantly higher in HCC patients with poorly differentiated tumours than in those with well-differentiated and moderately differentiated tumours.

In five cases in the HCC group, clinical response to adriamycin treatment was marked. Decrease in the tumour size (by more than 50%) was detected by u/s (data not shown). Seven cases showed a partial response to adriamycin, as indicated by a marked decrease in liver enzyme levels (data not shown).

Discussion

The prognosis for patients with HCC remains dismal despite the many advances made in its clinical study. Thus, the present study aimed to assess serum levels of TNF-α, sP-selectin, GGT, GST and AFP in HCC and SHF groups in order to understand the mechanism of HCC pathogenesis and its relationship to these parameters and the severity of the disease. The levels of all studied parameters were significantly increased in HCC patients before chemotherapy compared to the SHF and control groups,

Table 2. Spearman correlation (r) and its *P* value for all studied parameters related to the tumor size in the HCC group.

	TNF-α		sP-selectin		GGT		GST		AFP	
	r	*P*	r	*P*	r	*P*	r	*P*	r	*P*
Tumour size	0.253	0.481	0.198	0.584	−0.149	0.680	0.480	0.160	−0.038	0.926

Table 3. Relationship between the studied parameters and the pathological differentiation in the HCC patients group.

Diffentiation	TNF-α	sP-selectin	GGT	GST	AFP
Well (*n*=3)	258.7 ± 19.0	299.9 ± 13.9	96.8 ± 7.4	33.5 ± 0.4	13.8 ± 2.8
Moderate (*n*=7)	364.9 ± 38.5	311.5 ± 7.6	194.3 ± 17.4	62.6 ± 9.3	15.5 ± 3.3
Poor (*n*=5)	365.1 ± 36.0	313.4 ± 13.9	234.5 ± 4.5	125.7 ± 17.2	72.1 ± 29.4
	NS	NS	S	S	NS

S: significant, NS: non-significant.

and they revealed that TNF-α, sP-selectin, GGT, GST and AFP levels may have a diagnostic role in HCC patients.

Levels of TNF-α, sP-selectin and GST were significantly decreased after three cycles of treatment with adriamycin, compared to those in patients before treatment. Reduction in all parameters during treatment mirrored the response to chemotherapy and may be due to hepatic retention, the direct effect of adriamycin on cells, or both. This may indicate the validity of using these parameters in the follow-up of HCC patients treated with adriamycin.

In patients with poorly differentiated tumour, it was found that GGT and GST were significantly increased, while TNF-α, sP-selectin and AFP levels were not. This shows a good correlation between the levels of both GGT and GST and pathological differentiation in HCC.

Viral hepatitis status in HSF and HCC might explain the increased TNF-α production in those patient groups. It is generally accepted that HBV and/or HCV are major causes of chronic liver disease (e.g., chronic hepatitis and cirrhosis as well as HCC).[29] Larrea *et al*.[30] showed that hepatitis is associated with increased transcriptional expression of the TNF-α gene in the liver, with resultant high TNF-α serum levels. Several reports postulate that the production of TNF-α in the liver takes place in non-parenchymal cells and in hepatocytes.[30] In addition, Chisari[31] observed that in HBV- and/or HCV-infected patients the cytotoxic T lymphocytes (CTLs) control viral replication by direct cytotoxic activity of infected cells and via the production of IFN-γ and TNF-α.

In addition, it seems possible that oxidative distress might be involved in the induction of TNF-α in HCC and SHF patients.[32] Another possibility is its release from malignant or non-malignant cells (e.g., on activation of T cells or macrophages).[33]

In the present study, elevated levels of sP-selectin in both benign and malignant groups may be attributed to many factors. Some of these factors were studied in this work, such as TNF-α which induces the expression of P-selectin, affects the release of P-selectin from cells and seems to be important for hepatic injury in HCC or SHF patients. Also, elevated levels of serum sP-selectin may indicate platelet and endothelial cell activation/damage in those patient groups. However, it is not known whether the sP-selectin is secreted rather than shed from platelets or endothelial cells.[34] The increased levels of TNF-α and sP-selectin in the HCC patients more than in SHF patients can be attributed to the more intense inflammation (as indicated by C-reactive protein levels; data have not been shown) and severity of the disease which may be occurring in HCC patients.

The present study confirmed the value of GST in diagnosis and monitoring of HCC.[22,27] The presence of a significant, direct correlation between GST and GGT levels and tumour differentiation in HCC patients, and the absence of a correlation between GGT and GST and tumour size in the same patient group, suggests that production of GST and GGT is related to the biological characteristics of the tumour cells and not to tumour size.

The diagnostic value of serum AFP has been both confirmed[35–37] and refuted[21] by other workers. Also, the lack of any correlation between serum AFP level and both pathological differentiation and tumour size in HCC patients has been confirmed elsewhere.[22]

In conclusion, by excluding other inflammatory diseases and benign lesions, all parameters studied here can be used in HCC diagnosis. However, only serum TNF-α, sP-selectin and GST appear beneficial in monitoring chemotherapy in hepatitis-induced HCC. Clearly, further study, including a larger number of cases and use of different therapeutic modalities, is required to confirm the results presented. □

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