**Carcinogenesis** vol.23 no.1 pp.123–130, 2002

# Overexpression of glucose-regulated protein 94 (Grp94) in esophageal adenocarcinomas of a rat surgical model and humans

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**A rat surgical esophageal adenocarcinoma (EAC) model induced by esophagogastroduodenal anastomosis was recently established in our laboratory. This model mimics mixed reflux of gastric and duodenal contents in human patients and produces EAC without treatment with any carcinogen. We compared the protein expression pattern between rat EAC and normal tissues by 2-dimensional protein gel electrophoresis. The overexpressed protein spots of the tumor sample were cut out and analyzed by matrix-assisted laser desorption/ionization mass spectrometry. Several stress proteins (Grp94, Grp78, calnexin, Hsp90β and ER61) were identified by this method. Western blotting and RT–PCR further confirmed overexpression of Grp94 in rat EAC. Immunohistochemical staining also revealed expression of Grp94 in the epithelial cells of columnar lined esophagus and EAC. Similar to the rat model, well-differentiated human EAC and gastric cardia adenocarcinomas were also found to overexpress Grp94, but esophageal squamous cell carcinomas did not. We also characterized apoptosis, cell proliferation and oxidative DNA damage in the rat tissues. Since Grp94 is known to inhibit apoptosis by maintaining intracellular $Ca^{2+}$ homeostasis, our data suggest a possible correlation between oxidative stress, Grp94 overexpression and apoptosis regulation in esophageal adenocarcinogenesis.**

## Introduction

Esophageal adenocarcinoma (EAC) is the most rapidly increasing cancer in this country and has received considerable attention during the past two decades. The incidence of this deadly disease is increasing at a yearly rate of 4–10% in the USA (1,2). The 5 year survival rate, ~10%, has not increased significantly during the past 20 years (3). Therefore, it is important to understand the etiology and pathogenesis of this disease in order to prevent it at an early stage. Reflux of gastric and duodenal contents into the esophagus is known to be a major risk factor for human EAC. An average of ~10% of reflux esophagitis patients will develop columnar lined esophagus (CLE) (4,5). Since columnar epithelium is more resistant to chemical stress than squamous epithelium, CLE is believed to be an adaptive response to the harmful reflux (6). The risk of CLE patients developing EAC is 30–125 times greater than the general population (7). The most important intermediate marker for malignant transformation in CLE is severe dysplasia.

It is clear now that EAC arises as a result of tumor initiation and progression through hyperplasia, benign CLE without dysplasia, CLE with varying grades of dysplasia, carcinoma *in situ* and invasive EAC (8,9). In this process there is a progressive accumulation of genetic aberrations, leading to one or a few clones with a malignant tendency. Recently, Barrett *et al.* dissected out the clonal ordering of neoplastic lineages in human EAC. By focusing on *p53* gene mutation, *p16* gene mutation, non-random LOH, p16 methylation and ploidy, many intermediate clones with a tendency to progress to cancer were detected, suggesting multiple pathways leading to EAC. Only some of these clones persisted and developed into EAC, while others were delayed or stopped in their progression (10).

Animal models usually provide important information on human disease. We recently established a novel surgical model for EAC, known as esophagogastroduodenal anastomosis (EGDA), to mimic the combined reflux of gastric and duodenal contents into the esophagus. Similar to human EAC, EGDA animals developed sequential pathological changes from reflux esophagitis, to CLE (also known as Barrett's esophagus), CLE with dysplasia and, finally, EAC. Columnar cells in the esophagus are believed to originate from the duodenum in this model. This staged process of carcinogenesis provides a good system for mechanistic studies (11). With this model we also demonstrated that i.p. iron supplementation greatly increased the incidence of EAC. Iron deposition was observed in the areas with esophagitis, especially at the squamo-columnar junction, where all the EACs arose. Overexpression of inducible nitric oxide synthase and nitrotyrosine were seen in the rat esophagus after surgery and iron supplementation (12). Oxidative damage to DNA, lipid and protein were significantly higher in the esophagus than in non-operated control rats. CLE columnar cells are believed to be the targets of oxidative damage, since they overexpress heme oxygenase 1 and metallothionein, both known to be oxidative damage-responsive genes. We believe that rat esophageal adenocarcinogenesis was driven by oxidative stress secondary to chronic inflammation (12,13).

To further investigate the molecular changes underlying rat esophageal adenocarcinogenesis after EGDA, we compared the protein expression spectra in rat EAC and normal tissues (esophagus and duodenum) using 2-dimensional gel electrophoresis (2-DE) combined with matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS). This high throughput technique allows comprehensive profiling of post-transcriptional changes (14). We intended to discover differentially expressed genes, to test our hypothesis as to the role of oxidative damage in the formation of EAC and to identify possible biomarkers for early detection. This communication

**Abbreviations:** BrdU, bromodeoxyuridine; CLE, columnar lined esophagus; 2-DE, two-dimensional gel electrophoresis; 2′-dG, 2′-deoxyguanosine; EAC, esophageal adenocarcinoma; EGDA, esophagogastroduodenal anastomosis; Grp94, glucose-regulated protein 94; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; 8-OHdG, 8-hydroxydeoxyguanosine; PBS, phosphate-buffered saline.

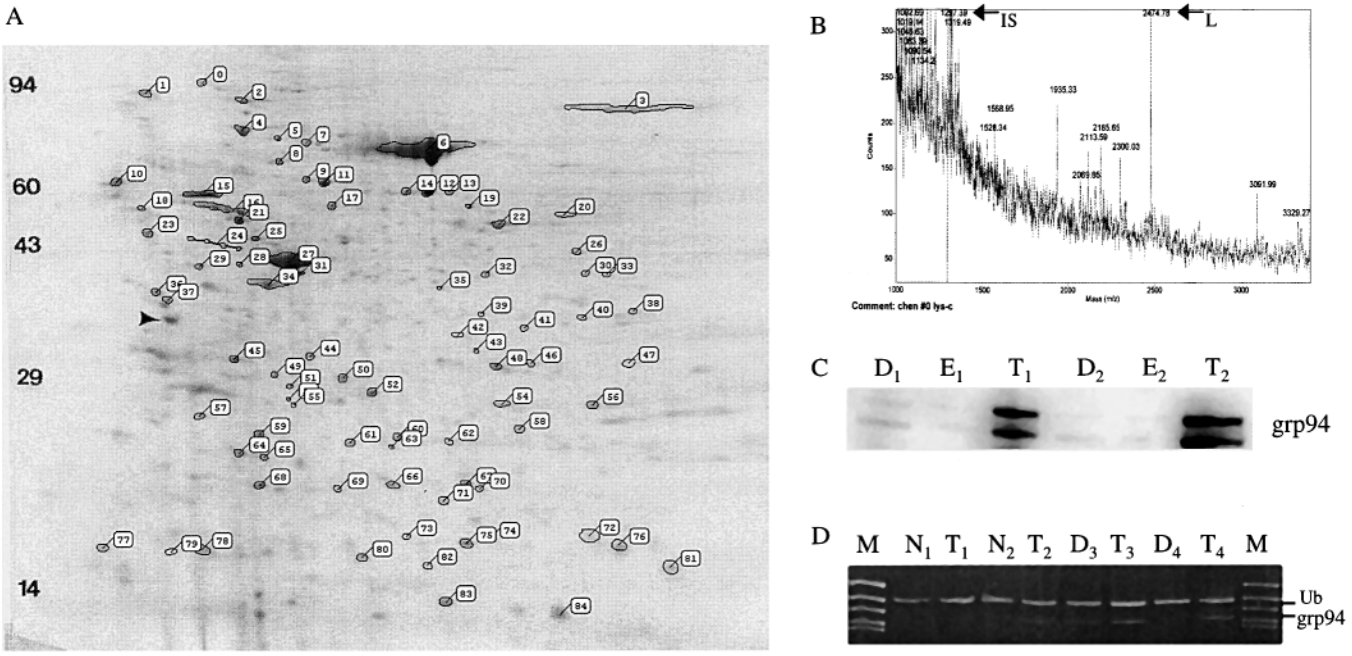


**Fig. 1.** Identification of Grp94 overexpression in rat EAC by 2-DE and MALDI-MS. (**A**) Protein spots on a 2-DE gel of one tumor sample are numbered. The black arrowhead points to the internal standard on the 2-DE gel. (**B**) The spectrum of one protein spot (spot 0) shows the presence of a few major mass peaks, which correspond to Grp94 in the SwissProt database. IS, internal standard; L, Lys-C. (**C**) Western blotting and (**D**) RT–PCR further confirmed overexpression of Grp94. An aliquot of 20 μg protein sample was loaded into each lane. D, duodenum; E, esophagus; T, EAC; M, DNA size marker V (Boeringer Mannheim).

describes a study on glucose-regulated protein 94 (Grp94). Characterization of other differentially expressed proteins is in progress.

## Materials and methods

*Material*

All the tissue samples were from a previous experiment with the EGDA model (11). The animals had EGDA and developed visible EAC. All tumors were pure well-differentiated mucinous EAC. All animals were injected with bromodeoxyuridine (BrdU) (50 mg/kg) 2 h before being killed. Frozen samples and formalin-fixed tissues were used for this experiment.

*2-DE, MALDI-MS and western blotting*

Frozen tissue samples (EAC, esophagus and duodenum) from two animals were rinsed rapidly with ice-cold phosphate-buffered saline (PBS), then homogenized on ice in 1.5 ml lysis buffer containing DNase/RNase and protease inhibitors (10 mM Tris, pH 7.4, 0.3% SDS, 50 μg/ml RNase, 100 μg/ml DNase, 5 mM $MgCl_2$). One milliliter of SDS boiling buffer (5% SDS, 5% β-mercaptoethanol, 10% glycerol, 60 mM Tris, pH 6.8) was then added to the homogenate and boiled for 10 min.

2-DE was performed according to the method of O'Farrell (15). Isoelectric focusing was carried out in glass tubes of inner diameter 2.0 mm, using 2% pH 4–8 ampholines (BDH, from Gallard Schlesinger, Long Island, NY) for 9600 V h. Fifty nanograms of an IEF internal standard, tropomyosin (mol. wt 33 000, pI 5.2), was added to each sample (50 μg protein) prior to loading. After equilibration in SDS sample buffer (10% glycerol, 50 mM dithiothreitol, 2.3% SDS, 0.0625 M Tris, pH 6.8), each tube gel was sealed to the top of a stacking gel overlying a 10% acrylamide slab gel (0.075 mm thick) and SDS slab gel electrophoresis was carried out for ~4 h at 12.5 mA/gel. All gels were silver stained for identification of differentially expressed proteins.

Duplicate gels were obtained for each sample as described above; one gel from each pair was scanned. Spots which appeared by eye to be different between the EAC and duodenum or between the EAC and esophagus, were outlined. The images were analyzed using Phoretix 2D Full software 4.0 such that differing spots and all major unchanging spots were outlined, quantified and matched on all the gels. In cases where protein spots were missing from some gels but present in others a small area of background was outlined appropriately to facilitate matching.

Twelve protein spots of interest were cut out, washed, dried and digested in-gel with 40 μl of digestion buffer containing 0.1 μg endoproteinase Lys-C (Boehringer Mannheim, Indianapolis, IN). The dried digest was then dissolved in 4 μl of matrix solution containing an internal standard (angiotensin) and 0.8 μl was spotted on the sample plate. MALDI-MS analysis was performed on the digest using a PerSeptive Voyager DE-RP mass spectrometer (PerSeptive Biosystems, Framingham, MA) in linear mode (16). Protein corresponding to the peptide masses detected was searched using an online database (http://www.ExPaSy.ch/).

SDS–PAGE and western blotting was performed according to a previously published protocol (17). Twenty micrograms of protein sample was loaded onto each lane. A polyclonal rabbit anti-Grp94 antibody diluted 1:2000 (Stressgen, Victoria, Canada) was used for detection of Grp94. The intensity was analyzed by ECL autoradiography and quantified using Image-Pro Plus software.

*Isolation of total RNA and RT-PCR*

Several pairs of total RNA samples were extracted from the EAC, esophagus and duodenum of the same rats with a PureRNA Isolation Kit from Clontech Laboratories (Palo Alto, CA). Yield and purity were assessed by measuring $A_{260}$ and $A_{280}$ and use of a denaturing agarose gel. The total RNA samples were then treated with DNase and used for RT–PCR. Reverse transcription was performed with a kit from Clontech (Advantage RT-for-PCR kit). Two sets of primers were designed for PCR: Grp94, forward, 5′-TACTATGCCAGTCAGAAGAAAACG-3′, reverse, 5′-CATCCTTTCTATCCTGTCTCCATA-3′ (product 198 bp); ubiquitin, forward, 5′-CGCTGGGCGGTTTGTTCCTCCATC-3′, reverse, 5′-TCCGGATGTTGTAGTCAGAGAGGG-3′ (product 231 bp). The PCR reaction contained 2 μl of cDNA from the RT reaction, 1× PCR buffer, 200 μM dNTPs, 1.5 mM $MgCl_2$, 2 μM each of the four primers, 0.025 U/μl AmpliTaq Gold DNA polymerase (Perkin Elmer, Foster City, CA). PCR conditions were as follows: 95°C for 10 min; 25 cycles of 94°C for 45 s, 60°C for 45 s, 72°C for 2 min; 72°C for 7 min. The PCR products were run on a 4% agarose gel.

*Immunohistochemistry of Grp94 and BrdU*

The avidin–biotin–peroxidase complex method (Elite ABC kit; Vector Laboratories, Burlingame, CA) was used for immunohistochemical staining for Grp94 and BrdU, according to the product manual. A polyclonal rabbit anti-Grp94 antibody diluted 1:500 (Stressgen) and a rat monoclonal anti-BrdU antibody at 2.5 μg/ml (Harlan Bioproducts for Science, Indianapolis, IN) were used. Frozen sections were briefly fixed for Grp94 immunostaining. Paraffin sections were pre-treated with 4 N HCl for 7 min for BrdU immunostaining. Negative controls were established by replacing the primary antibody with PBS and normal serum. Proliferative index was determined by calculating the ratio of

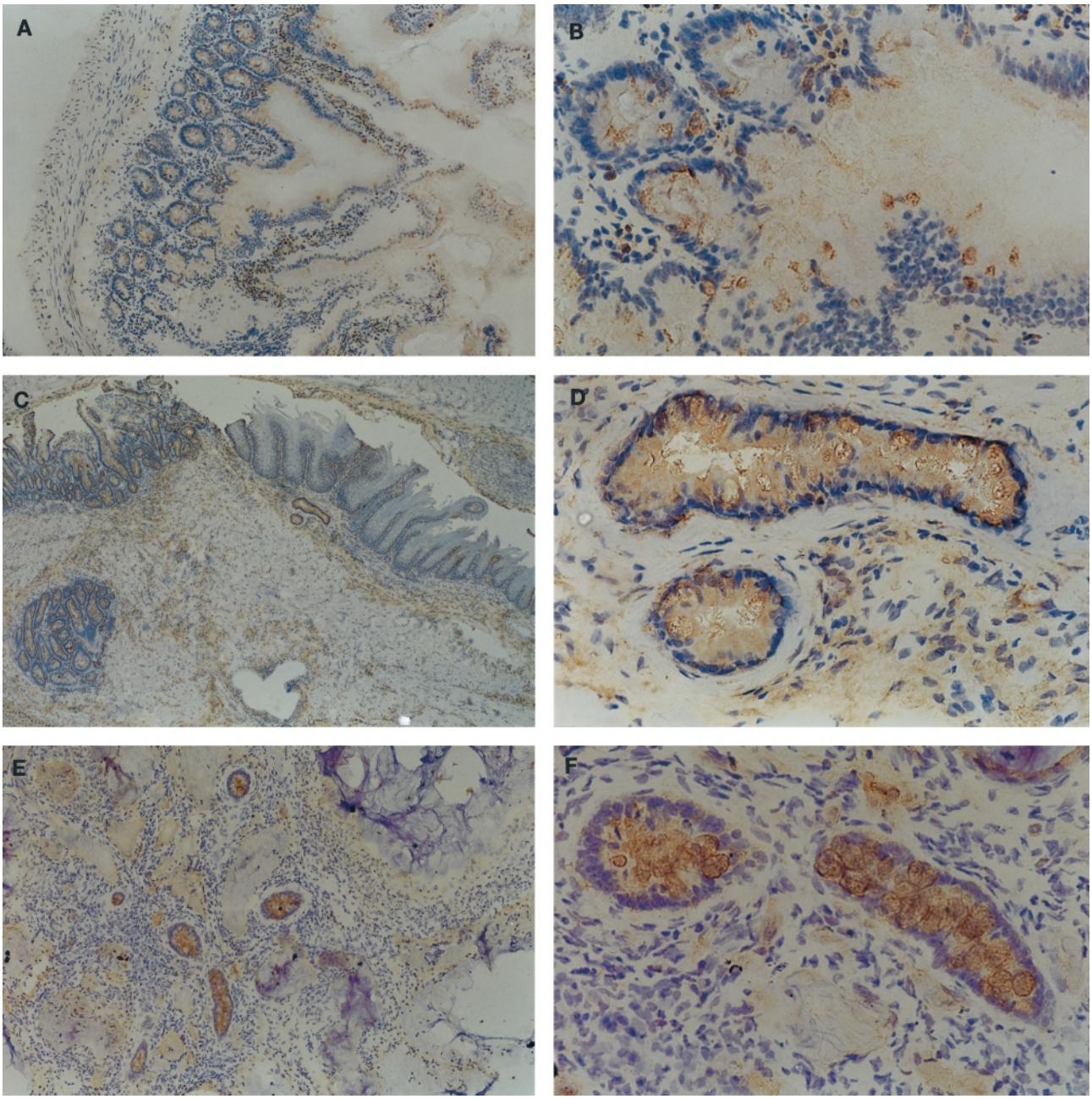


**Fig. 2.** Immunohistochemistry of Grp94 in duodenum (**A** and **B**), CLE (**C** and **D**) and EAC (**E** and **F**). (B), (D) and (F) (×400) are enlargements of (A), (C) and (E) (×100). Cells expressing Grp94 have their cytoplasm stained dark brown.

the number of BrdU-positive columnar cells to the total number of columnar cells. Sections from 10 rats and two slides for each rat were counted with ImagePro Plus software.

*In situ apoptosis detection: TdT-mediated biotin–dUTP nick end-labeling*

A TACS 2 TdT-DAB kit (Trevigen, Gaithersburg, MD) was used with the protocol provided by the manufacturer with minor modifications. Apoptotic index was determined by calculating the ratio of the number of positively stained columnar cells to the total number of columnar cells. Sections from 10 rats and two slides for each rat were counted with ImagePro Plus software.

*8-Hydroxydeoxyguanosine (8-OHdG) determination*

For the analysis of 8-OHdG, genomic DNA was extracted from the duodenum or the EAC with a Wako Extractor WB kit (Wako Chemical, Richmond, VA), hydrolyzed and analyzed with a HPLC system equipped with a reverse phase column (PFP column, 60 A, 5 μm; Princeton Chromatography, Princeton, NJ), an electrochemical detector (400 mA, Coulchem II; ESA Co., Chelmsford, MA) and a UV detector (280 nm, Waters 440; Waters Millipore). The amount was expressed as the molar ratio of 8-OHdG to 2′-deoxyguanosine (2′-dG) (8-OHdG per $10^5$ 2′-dG). Commercial calf thymus DNA (3 8-OHdG residues per $10^5$ 2′-dG residues; Sigma) was included every time for quality control. Samples from 10 rats (duodenum and EAC) were measured.

*Expression of Grp94 in human samples of normal esophagus, esophageal squamous cell carcinoma, EAC and gastric cardia adenocarcinoma*

Frozen surgically resected human samples of EAC (two cases), gastric cardia adenocarcinoma (two cases) and esophageal squamous cell carcinoma (two cases) were obtained from the Tissue Retrieval Service, Cancer Institute of New Jersey. Hematoxylin and eosin staining of the frozen sections confirmed the diagnosis. Care was taken to scratch tumor cells from frozen sections for extraction of total RNA and preparation of protein samples. The same amounts of total RNA were used for reverse transcription and PCR. The procedures were the same as those used for the rodent samples. Two sets of primers were used for PCR: human Grp94, forward, 5′-TACTATGCGAGTCAGAA-GAAAACA-3′, reverse, 5′-CATTCTTTCTATTCTATCTCCATA-3′ (product 198 bp). Primers for human glyceraldehyde 3-phosphate dehydrogenase were purchased from Clontech (primer mix included in the Advantage RT-for-PCR kit; product 983 bp). A rat monoclonal antibody against human Grp94 was used for western blotting (diluted 1:2000) (Stressgen) and immunohistochemistry

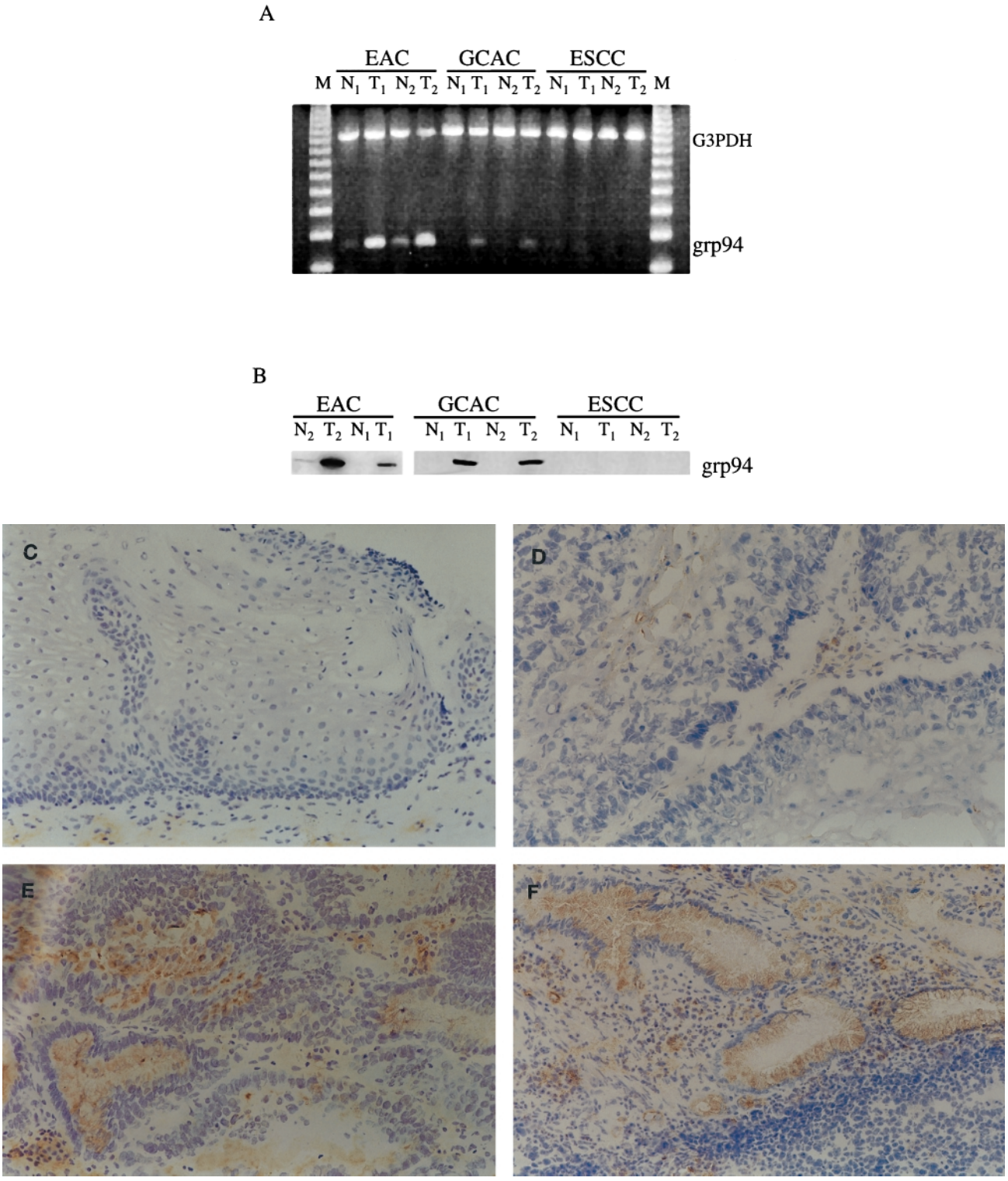


**Fig. 3.** Overexpression of Grp94 in human EAC and gastric cardia adenocarcinoma by (**A**) RT–PCR, (**B**) western blotting and (**C–F**) immunohistochemistry. M, 123 bp DNA ladder (Life Technologies, Grand Island, NY); T, tumor; N, accompanying normal esophagus or gastric cardia. Immunohistochemical staining of Grp94 was not observed in human normal esophagus (C, ×200) and esophageal squamous cell carcinoma (D, ×200). Grp94 was overexpressed in well-differentiated human EAC (E, ×200) and gastric cardia adenocarcinoma (F, ×200).

(diluted 1:500). Other procedures were the same as those used for the rodent samples.

## Results

### *Grp94 overexpression in rat EAC*

Aliquots of 50 μg protein from the tissue samples (EAC and nearby esophageal and duodenal epithelia) of two animals were run on 2-DE. After silver staining and laser scanning, the intensities of protein spots on 2-DE gels were quantified with imaging software (Figure 1A). The general method of computerized analysis for these pairs included automatic spot finding (pI and mol. wt) and quantification, automatic background subtraction and automatic spot matching in conjunction

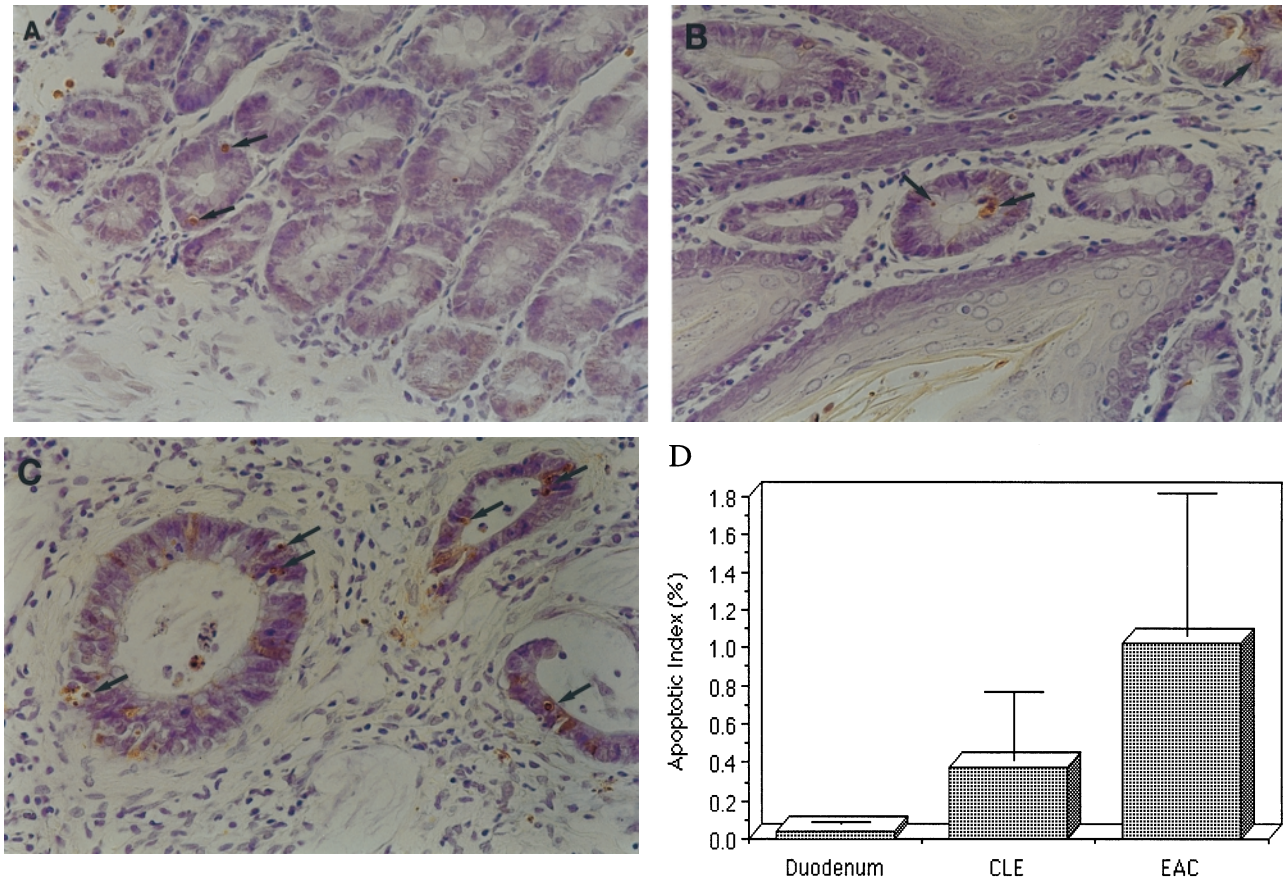


**Fig. 4.** *In situ* detection of apoptosis in (**A**) duodenum, (**B**) CLE and (**C**) EAC (×400). Arrows show apoptotic cells with their nuclei stained dark brown. (**D**) The apoptotic index was determined by counting the positive cells in all columnar cells.

with detailed manual checking of the spot finding and matching functions. Since comparisons were made between two different types of tissues (between the esophagus and EAC or between the duodenum and EAC), we only matched and quantified protein spots of high density and those suspected to be different between the tumor and the non-tumorous samples (84 spots).

Among these spots, 12 protein spots increased over 3-fold in the EAC in both pairs of samples. They were cut out from the 2-DE gel, digested in-gel and analyzed by MALDI-MS. After in-gel digestion with Lys-C, one of the spots (spot 0) showed six major peaks (Figure 1B). After searching the database, five of them (1935, 2113, 2185, 2300 and 3329) corresponded to an endoplasmic reticulum protein, named Grp94 (or gp96, ERp99, tumor rejection antigen 1 or endoplasmin; Swiss-Prot accession no. P08113). Several other differentially expressed protein spots were also identified as Grp78 (spot 4; Swiss-Prot accession no. P06761), calnexin (spot 1; Swiss-Prot accession no. P35565), heat shock protein 90β (spot 12; Swiss-Prot accession no. P34058), ER61 (spots 13 and 14; Swiss-Prot accession no. P11598), transferrin (spot 3; Swiss-Prot accession no. D38380), leukotriene $A_4$ hydrolase (spot 35; Swiss-Prot accession no. P30349), Rho GDP-dissociation inhibitor (spot 59; Swiss-Prot accession no. P19803), creatine kinase M chain (spot 30; Swiss-Prot accession no. P00564), vitamin D-binding protein (spot 17; Swiss-Prot accession no. P04276) and annexin V (spot 45; Swiss-Prot accession no. P14668). One spot (spot 52) was not identified due to lack of MS data in the database (MALDI-MS data not shown).

A polyclonal antibody against Grp94 was used for western blotting to confirm the result of Grp94 overexpression in rat EAC. Grp94 was overexpressed in the EAC as compared with the duodenum and esophagus (Figure 1C). Two closely spaced bands were observed on the western blot, probably due to differential phosphorylation or peptides covalently bound to Grp94, as reported previously (18).

RT–PCR validated the differential expression of Grp94 between the EAC and duodenum. Grp94 was co-amplified with ubiquitin as an internal control. In all cases Grp94 was overexpressed in the EAC (Figure 1D).

All of the above results were performed with tissue samples that contained many types of cells. To determine if Grp94 was overexpressed in tumor cells and its expression was correlated with disease progression, Grp94 expression was examined by immunohistochemistry on frozen sections from several animals. In the duodenum a moderate amount of Grp94 was detected in the cytoplasm of some surface epithelial cells of the intestinal villi. They appeared to be immature goblet cells (Figure 2A and B). In CLE most of the columnar cells expressed a moderate amount of Grp94 (Figure 2C and D). In EAC cells even higher expression was observed (Figure 2E and F). Therefore, Grp94 overexpression appeared to correlate with histological severity from normal epithelium to CLE to EAC, although some non-specific staining was observed. Grp94-positive cells were also observed in the stroma, possibly due to inflammatory cells.

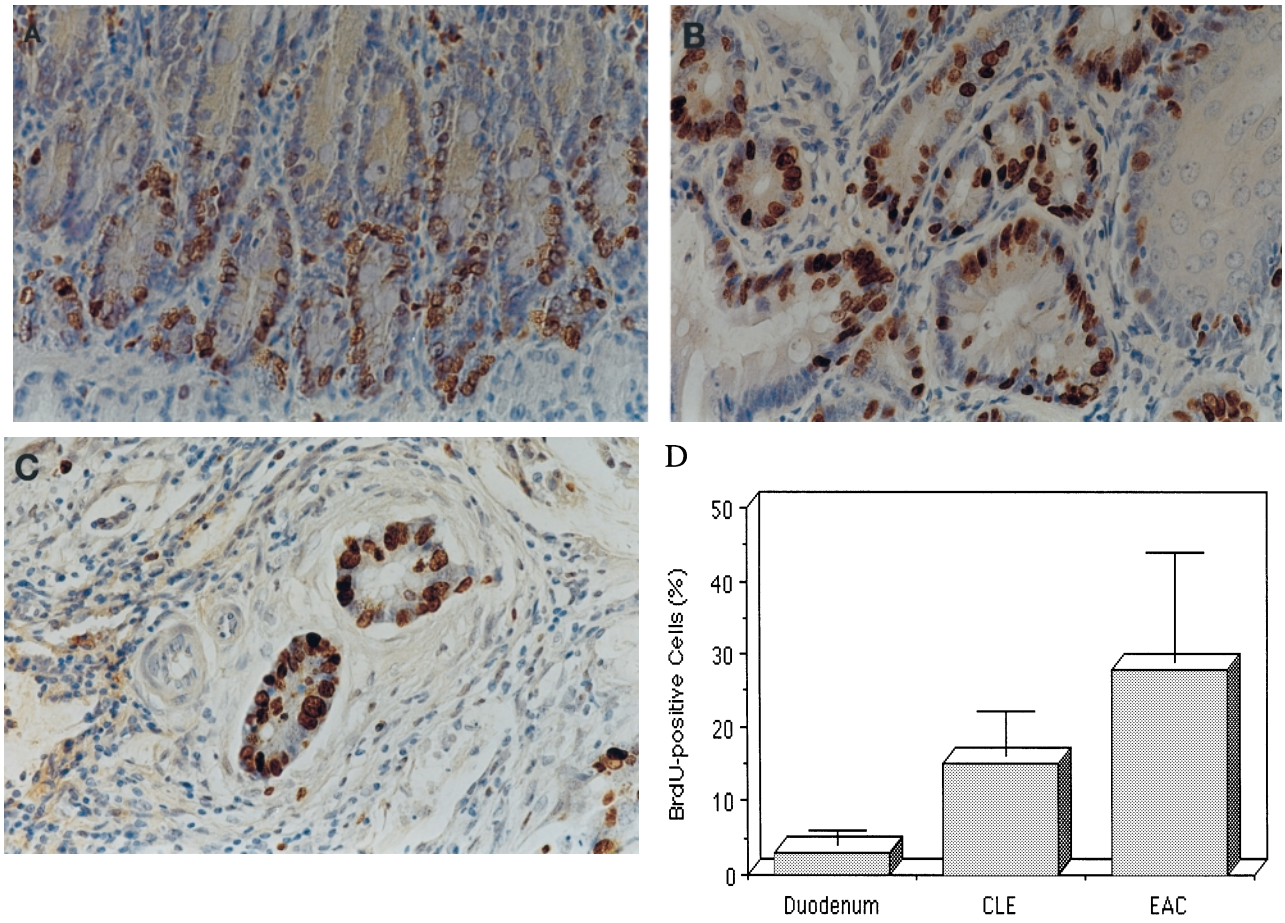


**Fig. 5.** BrdU immunohistochemistry for detection of proliferative cells in (**A**) duodenum, (**B**) CLE and (**C**) EAC (×400). Hyperproliferative cells have their nuclei stained dark brown. (**D**) The proliferative index was determined by counting the positive cells in all columnar cells.

*Grp94 overexpression in human EAC and gastric cardia adenocarcinoma, but not in esophageal squamous cell carcinoma*

To extend our studies into human adenocarcinomas, we examined expression of Grp94 in human EAC and the closely related gastric cardia adenocarcinoma (19). Frozen surgically resected samples of two cases each of EAC and gastric cardia adenocarcinoma were analyzed together with two cases of esophageal squamous cell carcinoma as a comparison. Tumor samples were compared with normal tissues at the margin of the resected esophagi (based on pathology). With RT–PCR overexpression of Grp94 was observed in both EAC and gastric cardia adenocarcinoma, but not in esophageal squamous cell carcinoma (Figure 3A). Western blotting yielded similar results (Figure 3B).

Immunohistochemistry was performed to assess the cellular localization of Grp94. There was a lack of staining in the normal esophagus (Figure 3C) and esophageal squamous cell carcinoma (Figure 3D). Two cases of well-differentiated human EAC were immunostained positive for Grp94. In both cases positive immunostaining for Grp94 was observed in the cytoplasm of tumor cells (Figure 3E). In gastric cardia adenocarcinoma Grp94 was expressed in the two human samples examined. Only well-differentiated, but not poorly differentiated, regions of the gastric cardia adenocarcinoma overexpressed Grp94 (Figure 3F). However, the limited number of human samples would not allow us to draw a conclusion concerning the relationship between differentiation and Grp94 expression.

*Apoptosis, hyperproliferation and oxidative DNA damage*

Grp94 has many different functions, one of which is to protect cells under oxidative stress against apoptosis (20,21). Apoptosis was analyzed by the TUNEL method and hyperproliferation by BrdU immunohistochemistry. Apoptosis was rare in the duodenum (Figure 4A), but significantly increased in CLE (Figure 4B) and EAC (Figure 4C). There was a statistically significant linear association between apoptotic index and increasing histological severity (Figure 4D) ($P < 0.05$). In the duodenum the apoptotic index was as low as 0.036%; it increased 10-fold to 0.37% in CLE and 30-fold to 1.02% in EAC.

Similar to the apoptotic index, proliferative index increased from the duodenum (3%, Figure 5A) to CLE (15%, Figure 5B) to EAC (28%, Figure 5C). There was a statistically significant linear association between proliferative index and increasing histological severity (Figure 5D) ($P < 0.05$).

In order to correlate hyperproliferation and apoptosis with oxidative stress, 8-OHdG was determined in our samples. In the EAC the amount of 8-OHdG per $10^5$ 2′-dG increased by 2-fold to 3.56 ± 1.47 from 1.54 ± 0.37 in duodenum ($P < 0.05$).

## Discussion

EGDA mimics the human situation in the rat by inducing mixed reflux of gastric and duodenal contents into the esophagus, thus producing CLE and, finally, EAC. According to our previous

study it generated 25.6% EAC at 40 weeks after surgery without treatment with any carcinogen. All tumors were purely well-differentiated mucinous EAC (11).

As reported previously, continuity of CLE from the duodenal epithelium suggested creeping substitution as the mechanism of CLE formation after EGDA (11). Therefore, we used both rat esophagus and duodenum as controls for 2-DE in this study. Grp94 overexpression was identified in the EAC cells (Figure 1). This result was confirmed by immunohistochemistry (Figure 2). Similar to the animal model, overexpression of Grp94 was observed in well-differentiated human EAC and gastric cardia adenocarcinoma, but not in esophageal squamous cell carcinoma (Figure 3). Grp94 was also overexpressed in some cancer cell lines and human tumor samples (21–24). Functionally, overexpression of Grp94 is related to tumorigenicity and tumor cell survival upon treatment. In rat colon adenocarcinoma cell lines more tumorigenic clones expressed more Grp94 under normal and stressed conditions (25). Grp94 transcription is generally activated by various stresses (26), such as treatment with lipopolysaccharide, phorbol esters, inflammatory mediators (24,27), irradiation (28) and chemotherapeutic agents (29,30), as well as conditions of hypoxia, acidosis, glucose deprivation and low nutrient supply (31). The rat EGDA model is a chronic inflammation-driven carcinogenesis model. Oxidative stress and inflammatory mediators are believed to play important roles (32). Overexpression of Grp94 is consistent with this concept. As a chaperone involved in protein folding in endoplasmic reticulum, Grp94 is also needed for cells that are active in production of secretory proteins, such as the mucinous adenocarcinoma cells in the EGDA model (33).

As a 94–100 kDa $Ca^{2+}$-binding protein ~50% homologous to its cytoplasmic counterpart Hsp90, Grp94 is the most abundant endoplasmic reticulum protein. Major functions of Grp94 include: (i) regulation of apoptosis; (ii) quality control of protein folding in endoplasmic reticulum by keeping proteins in a folding-competent state; (iii) presentation of antigenic peptides to MHC class I molecules; (iv) presentation of proteins to the proteolytic machinery by coupling to the proteosome (21,34). In the presence of various stresses the final fate of cells may mainly depend on the ability of the cells to resist the stress. Grp94 regulates cell fate by maintaining the intracellular $Ca^{2+}$ balance among cytosol, endoplasmic reticulum and mitochondria (20). In this study we observed a significant increase in overexpression of Grp94 from the duodenum to CLE to EAC. This is also well correlated with the increase in oxidative damage, as reflected in an increased level of 8-OHdG. However, overexpression of Grp94 was not associated with a decrease in apoptosis in rat EAC (Figure 4D). These results suggest that although Grp94 overexpression may protect the columnar cells in CLE from chronic inflammation-induced damage and allow them to develop into EAC, apoptosis still occurs if stress in the tissue is too strong.

Grp94 is one of the stress proteins covalently attached to peptides that may be tumor specific. Immunization with Grp94–peptide complexes derived from cancer cells elicits a potent T cell response against the cancer cells from which the Grp94–peptide complexes were purified (35). The efficacy of Grp94-mediated immunotherapy has been tested in several different animal cancer models (35–38). The pre-requirement for this approach is abundant expression of Grp94 in tumor tissue. In a human phase I trial of Grp94-mediated immunotherapy in human malignancy, 16 patients with various terminal malignant diseases who had failed to respond to prior established treatment regimens were immunized with autologous cancer-derived Grp94. No significant toxicity or autoimmune reactions were observed. Grp94 elicited MHC I-restricted, tumor-specific $CD8^+$ T lymphocytes and induced expansion of the NK cell population in some patients (39). Studies with our animal model in this direction may lead to a new therapy for human EAC patients.

## Acknowledgements

This study was supported by NIH grant CA75683 and facilities from NIEHS Center grant ES05022 and Cancer Center Support grant CA72720. C.S.Y. is a member of the Environmental and Occupational Health Sciences Institute (EOHSI) and the Cancer Institute of New Jersey (CINJ).

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*Received May 18, 2001; revised August 20, 2001; accepted September 17, 2001*