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Immunocytochemical and morphometric analysis of acinar zymogen granules in human acute pancreatitis

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Summary. In the present study fine structural changes of acinar zymogen granules were investigated in human acute pancreatitis. Pancreatic tissue was obtained at surgery from 6 patients, prepared for ultrastructural analysis, and stained immunocytochemically for trypsinogen. Stereological parameters of zymogen granules were evaluated. The density of the immunocytochemical labelling for trypsinogen was estimated over zymogen granules, the rough endoplasmic reticulum, Golgi apparatus and the acinar lumina. In acute pancreatitis the number of zymogen granules was diminished and their size reduced. The density of the labelling for trypsinogen was unchanged over zymogen granules but showed a significant reduction over the rough endoplasmic reticulum, Golgi apparatus, and the acinar lumina. In general the integrity of zymogen granules was well preserved. Focally degenerative changes of zymogen granules and large autophagosomes were observed. From the immunogold labelling a disturbance of enzyme synthesis and secretion was suggested. Evidence is given that a disruption of the zymogen granule membranes and a fusion with lysosomal bodies might contribute to the pathogenesis of human acute pancreatitis.

Key words: Human acute pancreatitis – Zymogen granules – Acinar cells – Electron microscopy – Immunocytochemistry – Morphometry

Introduction

It is generally agreed that human acute pancreatitis is the result of a rapid autodigestive process initiated in the pancreas (Chiari 1906; Doerr 1964; Creutzfeldt and Schmidt 1970; Becker 1981; Heitz and Klöppel 1984). It is, however, still a matter of debate how and where pancreatic enzymes become activated and contribute to tissue destruction.

There are only few ultrastructural investigations concerning the changes in acinar cells during the early phase of acute pancreatitis in man (Taura et al. 1975; Helin et al. 1980; Aho et al. 1982; Adler and Kern 1984; Bockman et al. 1986; Klöppel et al. 1986; Willemer and Adler 1989). They describe degeneration of cellular organelles, the presence of autophagic vacuoles, and the appearence of tubular complexes.

Contradictory results have been obtained regarding changes of zymogen granules in acinar cells. While in one study an increase in size and number of zymogen granules has been reported (Helin et al. 1980), we and others (Adler and Kern 1984; Bockman et al. 1986) noticed a decreased number of zymogen granules. Furthermore, only a little information exists on the fine structure of zymogen granules in acute pancreatitis. However, the disruption of their membrane might release hydrolases into the cytoplasm and result in disturbance of normal acinar cell function.

The present study was performed to investigate in detail the ultrastructure of acinar zymogen granules in acute pancreatitis. Using immunocytochemical and morphometric techniques the subcellular distribution of zymogen granules and a secretory protein (trypsinogen) in acinar cells was quantitated in tissues from patients suffering from severe acute pancreatitis.

Material and methods

Pancreatic tissue was obtained at surgery from six patients with acute pancreatitis (three men and three women). The age of

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Table 1. Stereological parameters for morphometric analysis of zymogen granules and immunocytochemical colloidal-gold labelling in human acute pancreatitis

Parameter	Dimension	Structure	Reference compartment (RC)	Abbreviation
1. Volume density	–	Zymogen Granules	Apical Cytoplasm	$V_{V(ZG/AC)}$
2. Mean size	μm^2	Zymogen Granules	–	$A_{(ZG)}$
3. Numerical area density	μm^{-2}	3.1. Zymogen Granules	Apical Cytoplasm	$N_{A(ZG/AC)}$
		3.2. Trypsinogen	RER/Golgi Apparatus	$N_{A(Tryp/RER,GA)}$
		3.3 Trypsinogen	Zymogen Granules	$N_{A(Tryp/ZG)}$
		3.4 Trypsinogen	Acinar Lumen	$N_{A(Tryp/AL)}$

the patients ranged from 25–75 years (mean 50 years). Pancreatitis was associated with alcoholism in three patients and biliary tract disease in the remaining three. Clinical onset of symptoms varied within a ten days interval.

Normal pancreatic tissue was obtained from two cadaver transplant donors and one patient operated on for a VIPoma of the pancreas. For electron microscopy samples were taken during surgery from the pancreas of the control group and from areas adjacent to necrosis in acute pancreatitis. The tissue specimen were immediately fixed in a mixture of 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M cacodylate buffer. After postfixation in 1% osmium tetroxide and standard methods for dehydration, tissue samples were embedded in Epon 812 and processed for immunocytochemistry.

Using the postembedding technique for immuno-electron microscopy (Rosano et al. 1974; Horisberger 1979), in the first incubation the ultrathin sections were exposed to the specific antiserum for 18 h at 4° C. The antiserum against trypsinogen was used at a 1:2000 dilution in PBS-buffer pH 7.4. After several washes in PBS-buffer the second incubation was performed with a goat-anti-rabbit antibody coated with colloidal-gold (15 nm diameter colloidal-gold-complex) (purchased from Janssen Pharmaceutica, Bearse, Belgium) for 30 min at room temperature.

Then the sections were washed several times in PBS-buffer and finally rinsed in distilled water. Ultrathin sections were stained with uranylacetate and lead citrate and examined using a Zeiss EM 109 electron microscope.

The antiserum against bovine trypsinogen was raised in rabbits by injecting 300 µg enzyme preparation (highest commercially avaible purity, Sigma Chemicals, Munich, FRG), dissolved in 0.5 ml phosphate buffered saline and 0.5 ml Freud's complete adjuvant. After repeated injections at four weeks intervals the rabbits were bled one week after the last injection.

The characterization of the antibodies and their application in immunocytochemical studies has been published elsewhere (Klöppel et al. 1986; Willemer and Adler 1989). The specificity of immunostaining for trypsinogen was determined by preabsorption of the antiserum with the appropriate antigen for 12 h at 4° C before application to the sections and incubation of the sections with the colloidal-gold coated antibody alone. The sections of normal human pancreas served as positive controls.

For morphometric investigations of the zymogen granules a total of 60 micrographs were taken from four ultrathin sections of each pancreas over the apical cytoplasm at a primary magnification of 12000 ×. To estimate the density of the colloidal-gold labelling for trypsinogen over the rough endoplasmic reticulum, Golgi-complex, and the acinar lumina, additional micrographs were taken (30 for each examined pancreas and compartment) at the same magnification (12000 ×).

The micrographs were selected throughout the sections over structurally intact acinar cells. They were defined by a well preserved subcellular architecture and the lack of apparent swelling of mitochondria or dilation of the rough endoplasmic reticulum.

Following standard stereological principles (for review see Weibel 1979 and Oberholzer 1983) each film negative was analysed using a semiautomatic computerized analysis system (Zeiss Morphomat 30, Oberkochen, FRG). As indicated in Table 1, volume density of zymogen granules, mean size of zymogen granules, and numerical area density of colloidal-gold labeled trypsinogen were calculated. For estimation of the volume density and numerical area density of zymogen granules the apical cytoplasm of acinar cells was choosen as reference compartment. Only micrographs that revealed a statistically homogen (isotropical) distribution of the granules within the apical cytoplasm were analysed.

The basal and perinuclear cytoplasm was defined as reference compartment to calculate the numerial area density of the immunogold labelling for trypsinogen over the rough endoplasmic reticulum and Golgi-apparatus (gold particles per μm^2). In addition the density of the immunogold labelling for trypsinogen was calculated over zymogen granules and acinar lumina. The statistical analysis for significance (p-values) was performed using the Mann-Whitney test (U-Test). A p-value of <0.01 was considered significant.

To characterize the pattern of the immunocytochemical staining for cathepsin B as a marker for lysosomal activity in acute pancreatitis tissue was fixed in 4% buffered formaldehyde and processed to paraffin sections. The polyclonal antibody against cathepsin B (sheep antiserum against human cathepsin B) was purchased from Camon Chemicals, Wiesbaden, FRG. Immunocytochemistry was carried out on subsequent deparaffinized section using the avidin-biotin-peroxidase complex method as has been described previously in detail (Klöppel et al. 1986; Willemer and Adler 1989). The antibody against cathepsin B was used at a 1:500 dilution in PBS-buffer pH 7.2. The specificity of immunostaining was determined by preabsorption of the antibody with the appropiate antigen.

Results

Morphometric analysis of zymogen granules revealed a markedly reduced volume density in acinar cells during acute pancreatitis (Tables 2 and 3; $V_{V(ZG/AC)}$). The diminution of the volume density of zymogen granules was due to a reduced total number ($N_{A(ZG/AC)}$) and size ($A_{(ZG)}$) compared with

Table 2. Results of morphometric analysis of zymogen granules and trypsinogen labelling in human acute pancreatitis and controls

Parameter (Abbrevation)	Acute pancreatitis						Controls		
	case 1	case 2	case 3	case 4	case 5	case 6	case 7	case 8	case 9
Zymogen Granules									
$V_{V(ZG/AC)}$	0.16	0.16	0.18	0.19	0.11	0.13	0.32	0.33	0.33
$A_{(ZG)}$	2.31	2.13	3.03	2.08	2.27	2.03	3.31	3.85	3.54
$N_{A(ZG/AC)}$	0.07	0.06	0.06	0.09	0.05	0.07	0.14	0.13	0.14
Trypsinogen labeling									
$N_{A(Tryp/RER,GA)}$	0.12	0.24	0.09	0.08	0.05	0.05	0.60	0.57	0.58
$N_{A(Tryp/ZG)}$	13.92	18.48	19.68	17.04	12.00	15.60	16.40	15.12	15.72
$N_{A(Tryp/AL)}$	0.56	0.47	0.51	0.52	0.49	0.50	10.40	12.40	14.61

Data expressed as mean values of each parameter and patient

Table 3. Combined results of morphometric evaluation and statistical analysis

Parameter (Abbrevation)	Acute pancreatitis (total)		Controls (total)		Niveau of significance*
	mean values	standard deviation	mean values	standard deviation	
Zymogen Granules					
$V_{V(ZG/AC)}$	0.15	0.07	0.32	0.09	$P<0.001$
$A_{(ZG)}$	2.31	0.77	3.56	1.32	$P<0.001$
$N_{A(ZG/AC)}$	0.07	0.03	0.14	0.04	$P<0.001$
Trypsinogen labeling					
$N_{A(Tryp/RER,GA)}$	0.10	0.05	0.60	0.15	$P<0.001$
$N_{A(Tryp/ZG)}$	16.10	6.12	15.70	4.40	$P>0.1$
$N_{A(Try/AL)}$	0.51	0.32	12.40	4.56	$P<0.001$

*The statistical analysis of significance (P-values) was performed using the Mann-Whitney test (U-test)

the control group (Fig. 1). The zymogen granules mostly appeared intact with a homogenous electron dense core and a normal pattern of immunogold labelling for trypsinogen. Focally, the zymogen granules were concentrated along the basolateral instead of the luminal plasma membrane of the acinar cell (Fig. 1). No figures of basolateral exocytosis and discharge of immunogold labeled content were seen in these areas. Exocytotic figures and coated pits at the luminal plasma membrane were less frequent but still found in acute pancreatitis. In addition, disorganisation and condensation of the submembraneous filamentous web was found at the luminal side of acinar cells. The microvilli appeared reduced in number and quite often intact zymogen granules were seen in the acinar lumina (Fig. 1).

In some zymogen granules and condensing vacuoles a decrease in the electron density and immunogold labelling intensity was observed from the periphery toward the center (Figs. 2 and 3a). Severe membrane destruction became obvious with consecutive fusion of condensing vacuoles as well as individual granules (Figs. 2 and 3a).

Focally, large autophagosomes containing zymogen granules and other cellular organelles were seen (Fig. 3b). Intracellular release of zymogen content and the formation of larger areas of amorphous floccular material occurred. This material still showed labelling with the trypsinogen antibody (Fig. 3c and 3d). Finally, apart from necrotic remnants of cell organelles, intact and dissolving zymogen granules were seen in the extracellular space (Fig. 4).

Immunocytochemical labeling for trypsinogen in normal pancreatic tissue was restricted to the different membrane bound compartments of the acinar cells (rough endoplasmic reticulum, Golgi apparatus, condensing vacuoles, zymogen granules) and the acinar lumina (Fig. 5a). An increasing gradient of the labelling was seen from the rough endoplasmic reticulum to the zymogen granules. Only few gold particles were observed over mitochondria, nuclei, and the extracellular space.

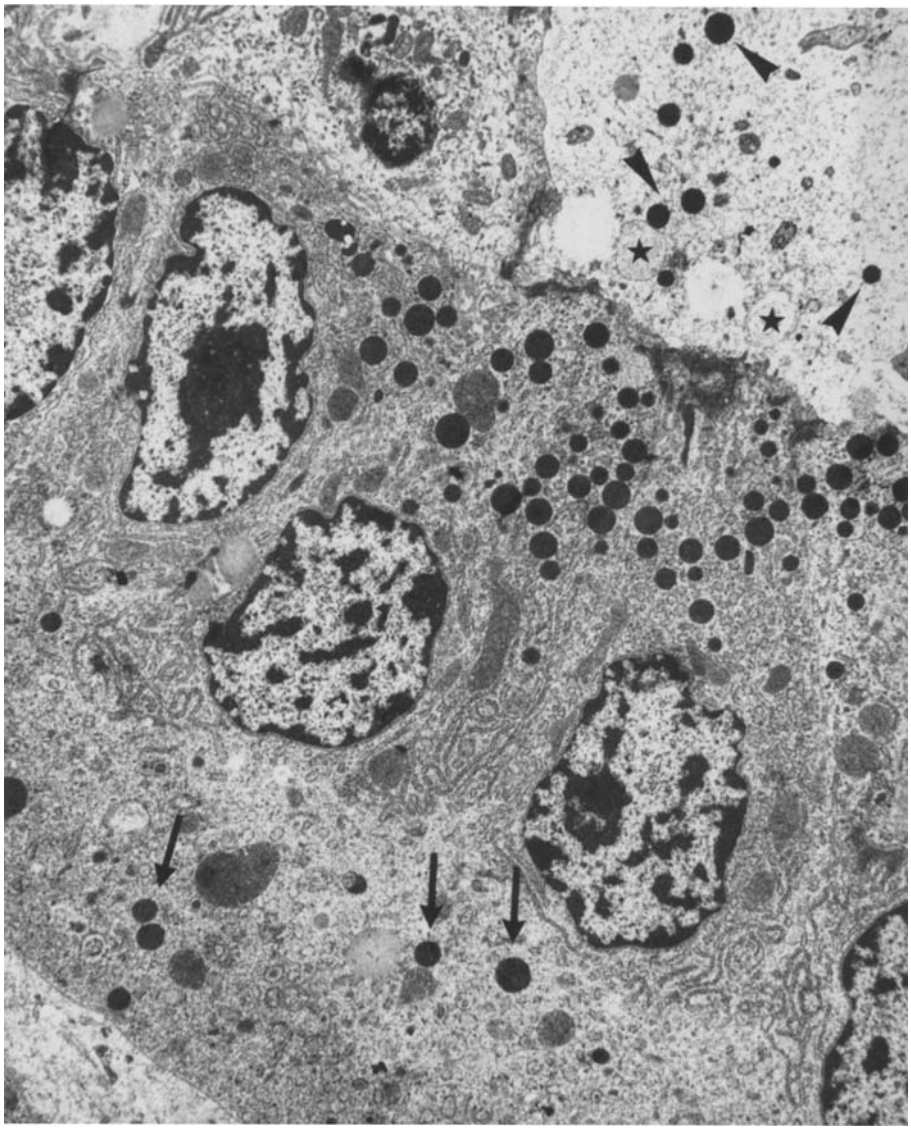

Fig. 1. Acinar cells in acute pancreatitis. Zymogen granules in the apical cytoplasm appear reduced in number and size. Few zymogen granules were found in the basal part of acinar cells (*arrows*). The acinar lumen is filled with few zymogen granules (*arrowheads*), membrane remnants (*asteriks*), and amorphous material (×9500)

When the antisera were incubated with the corresponding antigens (trypsinogen) before application to the sections, only few gold particles were seen over the different cellular organelles and the extracellular space. Similar results were achieved by incubation of the sections with the colloidal-gold coated antibody alone.

In acute pancreatitis the numerical area density for the labelling of trypsinogen ($N_{A(TRYP/ZG)}$) in zymogen granules was virtually unchanged compared to controls and remained almost constant in all patients examined (Tables 2 and 3). In contrast, a six-fold reduction in the labelling of the rough endoplasmic reticulum and the Golgi apparatus was found in acute pancreatitis. This was evaluated in terms of a decreased numerical area density of immunoreactive trypsinogen (Tables 2 and 3; $N_{A(TRYP/RER;GA)}$). In normal pancreatic tissue a dense colloidal-gold labelling for trypsinogen was observed over the acinar lumina (Fig. 5a). In contrast, acinar lumina in acute pancreatitis revealed a more than tenfold reduction in the density of gold particles bound to immunoreactive trypsinogen (Tables 2 and 3; $N_{A(TRYP/AL)}$) (Fig. 5b). In few acinar lumina fibrillar precipitations were found with an inhomogenous distribution of floccular electron dense and immunoreactive material (Fig. 6).

The immunocytochemical staining for cathepsin B showed a moderate reaction over the islets of Langerhans in acute pancreatitis and the controls (Fig. 7). The acinar parenchyma was virtually

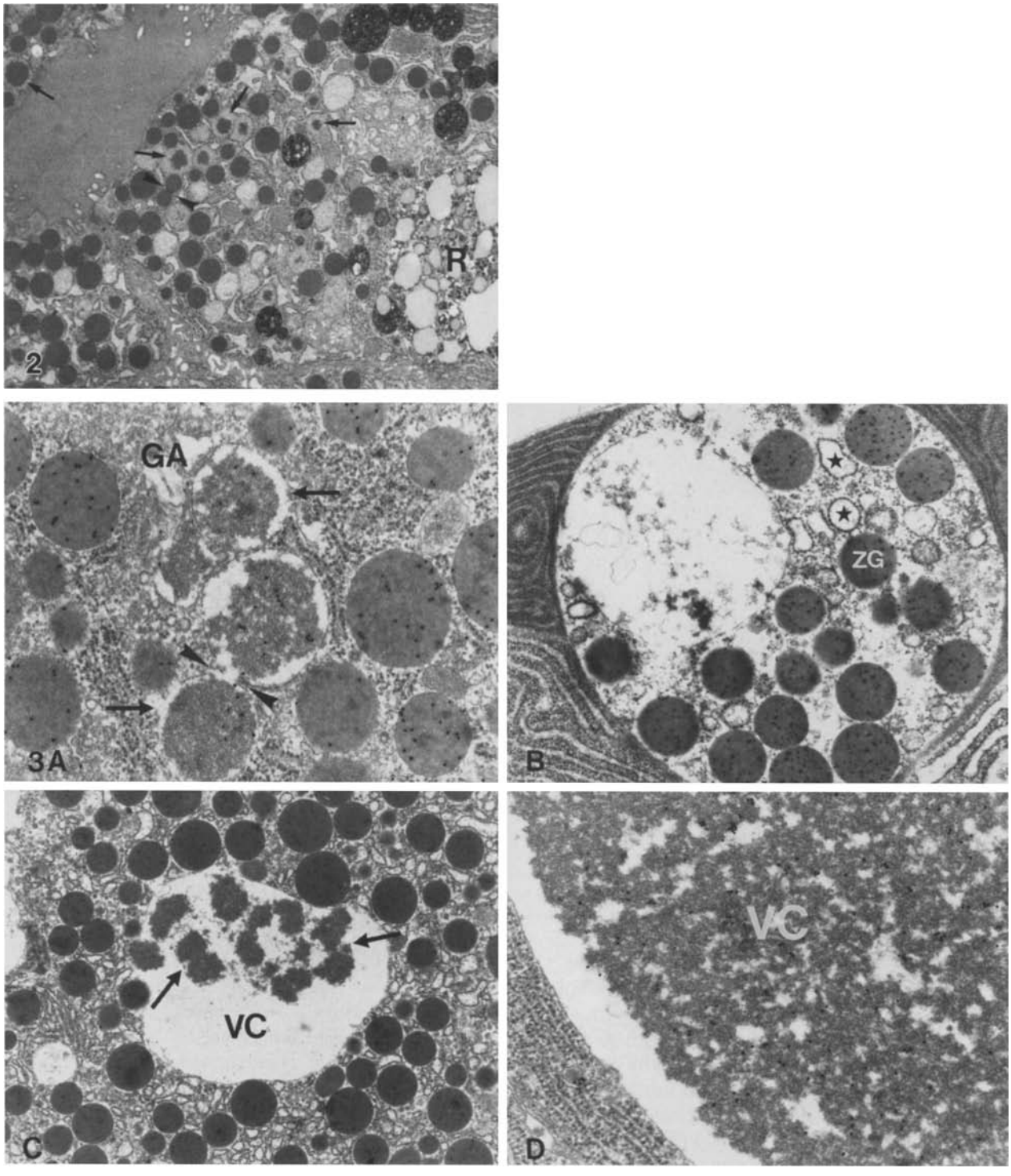

Fig. 2. Focal breakdown of zymogen granules. Loss of electron density of the core proceeding from the periphery to the center (*arrows*). Fusion of individual granules (*arrowheads*). Residual body (R) (×9100)

Fig. 3. (**A**). Disruption of the limiting membrane (*arrow*) and fusion of condensing vacuoles (*arrowheads*). Reduced immunoreactivity of the floccular appearing content. Golgi apparatus (GA). Trypsinogen (×20000). (**B**) Large autophagic vacuole containing zymogen granules (ZG), fragments of the rough endoplasmic reticulum (*asteriks*) and membrane remnants. Trypsinogen (×19600). (**C**) and (**D**) Formation of larger intracytoplasmic vacuoles (VC) containing fused zymogen content (*arrows*) still immunoreactive for trypsinogen. Besides severe cellular deterioriation becomes obvious with fragmentation of the rough endoplasmic reticulum (**C**). (**C**) (×12000), (**D**) (×20000)

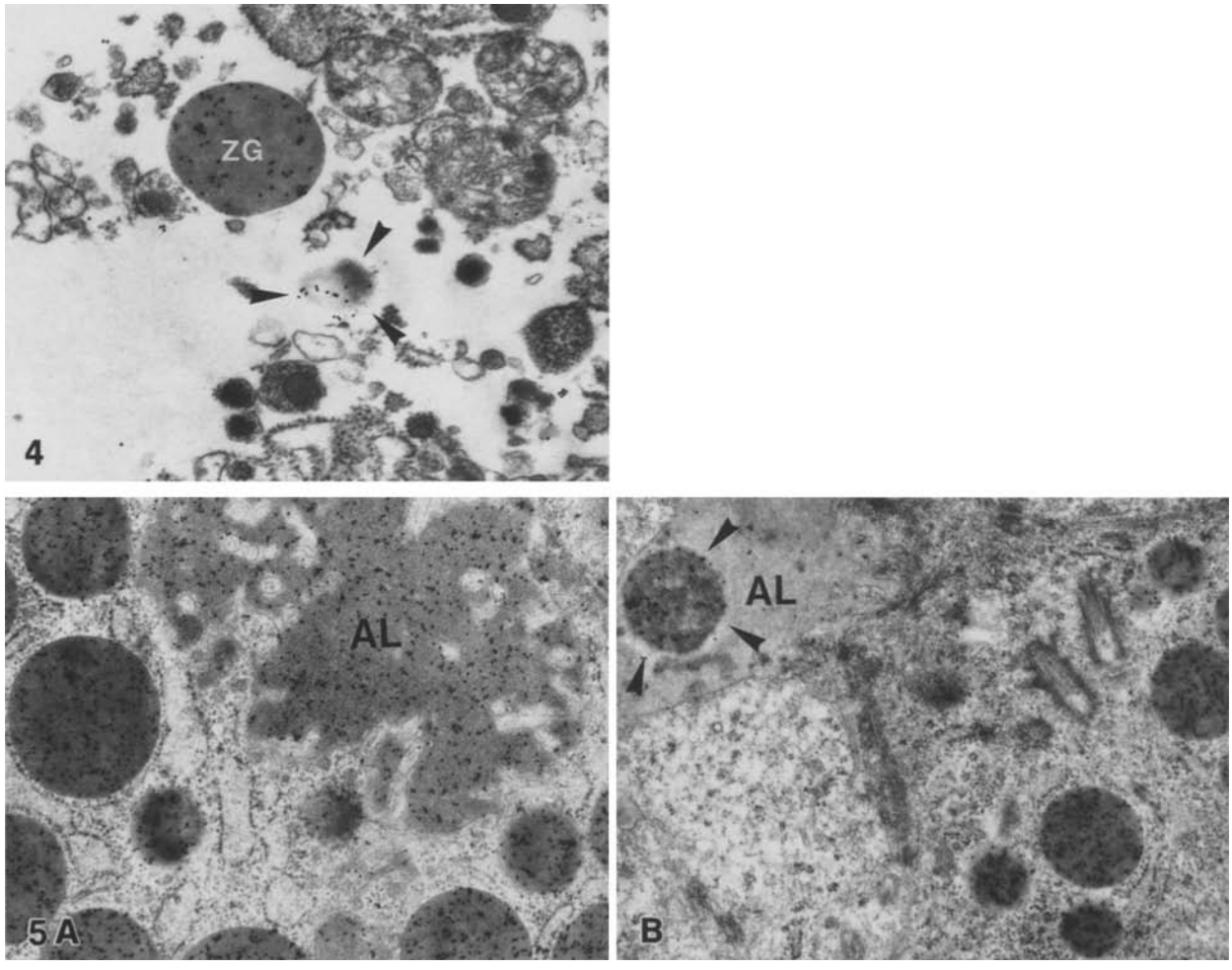

Fig. 4. Zymogen granules in the extracellular space. Remnants of necrotic cells and still intact zymogen granules (ZG) are found beside dissolving granules (*arrowheads*). Trypsinogen (× 20000)

Fig. 5. Immunocytochemical labelling for trypsinogen in the apical part of acinar cells. A dense labeling of gold particles is found over zymogen granules (ZG) and the acinar lumen (AL) in the control pancreas. (**A**) A markedly reduced density of gold particles over the acinar lumen is seen in acute pancreatitis. (**B**) A secretory granule (*arrowheads*) is found in the acinar lumen. The cytoplasm adjacent to zymogen granules reveals a very low labelling (× 25000)

unstained in the controls (Fig. 7a), while in acute pancreatitis an irregular distributed spotty immunoreactivity was found (Fig. 7b). Focally cytoplasmic vacuoles showed an intense staining reaction. Surrounding polymorphonuclear granulocytes and macrophages were heavily stained (Fig. 7b).

Discussion

The present stereological investigation on the pancreas of patients suffering from acute pancreatitis reveals a reduction in the number and size of zymogen granules in acinar cells without change of the density of colloidal-gold label for trypsinogen. Over the rough endoplasmic reticulum, the Golgi apparatus, and the acinar lumina the label for trypsinogen was markedly reduced. These findings on the distribution of zymogen granules and the immunocytochemical label for a secretory protein require careful interpretation.

First, the technique of immunogold labelling is based on immunological interactions and the quantitative data reflect amounts of antigenic material and not enzymatic activities. Second, alterations of antigenicity due to fixation and embedding, and an altered immunoreactivity of secretory proteins in acute pancreatitis have to be taken into account. Third, immunogold labelling cannot be interpreted in dynamic-kinetic terms. However, Bendayan et al. 1980 have shown that a reproduc-

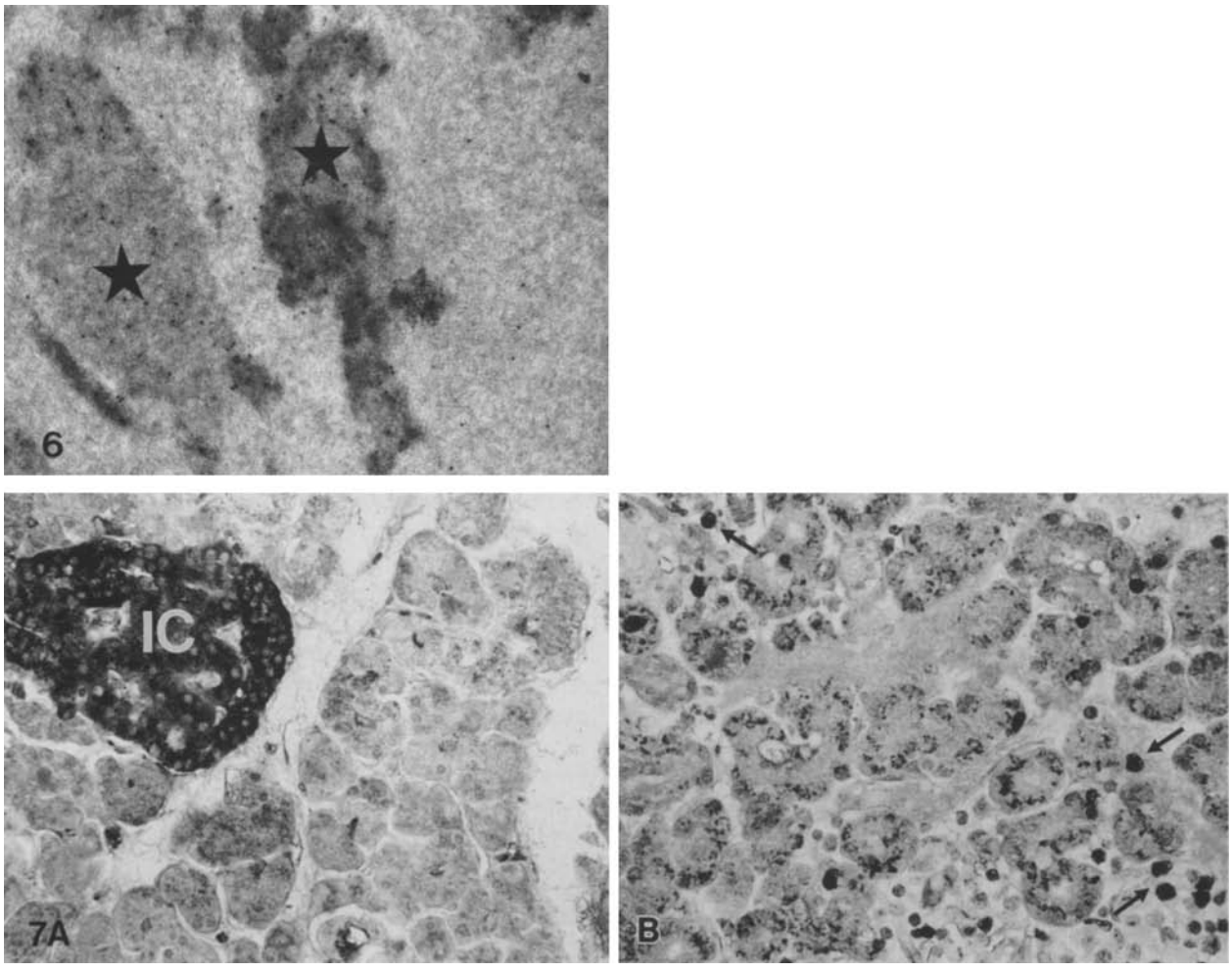

Fig. 6. Floccular distributed electrondense and immunoreactive material (*asteriks*) beside fibrillar precipitations in acinar lumina. Trypsinogen (×20000)

Fig. 7. Immunocytochemical staining for cathepsin B. **(A)** Normal pancreatic tissue displaying intense immunostaining of islet cells (IC), the surrounding acinar cells remain unstained (×250). **(B)** Severe acute pancreatitis: Immunostaining for cathepsin B shows a marked increased granular staining reaction in acinar cells. Surrounding inflammatory cells in the interstice are heavily stained (*arrows*) (×250)

ible quantitative, immunocytochemical localization of secretory proteins is possible in subcellular compartments of the acinar cell. They found that the number of gold particles reflect true differences in enzyme content, compared with known biochemical findings.

From these data we conclude that in human acute pancreatitis the synthesis of secretory proteins is impaired. In agreement with these morphological results, previous biochemical analysis from our laboratory indicated a decrease in protein synthesis in isolated lobules from patients with acute pancreatitis (Kern and Adler 1984). Further indirect support to our findings is provided by immunocytochemical studies performed at the light microscopic level which revealed reduced immunoreactivity of pancreatic enzymes in acinar cells in the vicinity of fat necrosis (Aho et al. 1983; Klöppel et al. 1984).

In general the zymogen granules were well preserved, and revealed a normal pattern of colloidal-gold labelling for trypsinogen even in severely damaged cells. Focally, however, major alterations in the fine structure of individual granules appeared including intracellular fusion and disruption of the granule membranes with consecutive release of immunoreactive material. In these areas cytoplasmic vacuoles containing immunoreactive material similar to zymogen granules were found. In addition, fusion of zymogen granules with lyso-

somal bodies resulted in the formation of large autophagosomes. No figures of basolateral exocytosis of zymogen granules or cytoplasmic vacuoles with extracellular discharge of immunoreactive material were found, although quite often zymogen granules were concentrated in the basal cytoplasm. This phenomenon has been reported in secretagogue-induced pancreatitis in the rat (Adler et al. 1982; Scheele et al. 1987) and was discussed as one of the mechanisms for interstitial enzyme release. However, in the present study zymogen granules were discharged into the interstitial space from ruptured acinar cells, in close proximity to necrotic areas.

From our fine structural findings we suggest that in acute pancreatitis intracellular activation of digestive enzymes could be mediated by two potential mechanisms. The first involves zymogen activation by lysosomal hydrolases (such as cathepsin B) in autophagosomes. This potential mechanism of trypsin activation has been demonstrated (Greenbaum and Hirshkowitz 1961). In support of this concept, fusion of lysosomes with zymogen granules and formation of large Golgi-derived vacuoles – containing lysosomal hydrolases and digestive enzymes – was found in diet-induced (Lombardi et al. 1975; Gilliland and Steer 1980; Koike et al. 1982) and secretagogue-induced pancreatitis (Adler et al. 1982; Lampel and Kern 1977; Adler et al. 1979; Watanabe et al. 1984; Saluja et al. 1985). In both experimental models of acute pancreatitis, the cocompartmentation of digestive and lysosomal enzymes in large intracytoplasmic vacuoles was believed to account for the activation of digestive enzymes within the acinar cells (Koike et al. 1982; Saito et al. 1987; Saluja et al. 1987). The importance of increased lysosomal activity in human acute pancreatitis was supported by the localisation and marked staining reaction of cathepsin B in acinar cells. The second potential mechanism of intracellular zymogen activation involves a focally occurring premature intrapancreatic auto-activation of digestive enzymes within the zymogen granules and a consecutive fragility of the surrounding membranes. In addition, a primary instability of the zymogen granule membranes due to an impaired process of maturation in the Golgi apparatus is possible.

In accordance with this concept, ultrastructural findings from Eppig and Leiter (1977) dealing with the pathogenesis of spontaneously occurring exocrine pancreatic insufficiency (EPI) syndrome in CBA/J mice are of interest. They found that the initial cytological manifestation of this syndrome was a progressive digestion of the zymogen granules, fusion of granules, granule membrane breakdown, and zymogen release into the cytoplasm. In their biochemical studies (Leiter et al. 1977) premature intracellular proteolysis by uncontrolled autoactivation of trypsinogen and chymotrypsinogen within the zymogen granules was shown to be the crucial mechanism.

Under certain circumstances conversion of inactive trypsinogen to active trypsin can be initiated autocatalytically by the zymogens themselves (Kassels and Kay 1973). It is, however not known, whether this mechanism could be relevant in human acute pancreatitis.

The findings reported here on surgical specimens cannot be expected to demonstrate the earliest changes which result in the development of human acute pancreatitis. Furthermore, no clear distinction can be made as to whether these changes occur in the initial state of pancreatic unjury or whether they occur as a consequence of pancreatitis, playing a role in the further progress of the disease. However, it must be stated that the complex changes of zymogen granules observed in the present study indicate an alteration of the compartmentation of pancreatic enzymes and a possible key role for secretory granules in the process of uncontrolled enzyme activation and autodigestion of the gland.

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