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Overall Proteolysis in Perfused and Subfractionated Chemically Induced Malignant Hepatoma of Rat: Effects of Amino Acids

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Control livers and chemically induced hepatoma-bearing livers of nonstarved rats were perfused cyclically with and without the addition of amino acids (known to suppress proteolysis) to the perfusate. Morphologic analysis of the fractional cytoplasmic volume of the lysosomal apparatus (dense bodies and autophagic vacuoles) demonstrated that the addition of amino acids to the perfusion medium inhibited autophagic sequestration of cytoplasm in both tumor and control hepatocytes, although the inhibition was stronger in control than in tumor hepatocytes. The fractional cytoplasmic volume of autophagic vacuoles (AVs) was larger in hepatoma cells than in control hepatocytes regardless of whether amino acids were added or not. The transition (degradation of sequestered cytoplasm) of AVs into dense bodies seems to be prolonged in malignant hepatoma cells. Assessment of rates of protein degradation both in the perfusion medium and in isolated lysosomes disclosed that proteolysis was much lower in tumor liver than in control liver. This can be explained by lower lysosomal enzyme activities in tumor cells, as was evident from tissue homogenate and isolated lysosomes. The addition of amino acids to the perfusate reduced total proteolysis from 1.73 to 0.78% per hour in control hepatocytes and from 0.49 to 0.33% per hour in tumor hepatocytes, i.e., inhibitions of 55 and 33%, respectively. Proteolysis as estimated from isolated lysosomes was also inhibited by amino acids added to the perfusion medium but the inhibition was more conspicuous in control (from 14 to 7.4%) than in tumor cells (from 5.2 to 3.6%). *In conclusion,* the results show that the relative cytoplasmic volume of AVs is higher but overall proteolysis lower in malignant hepatoma tissue than in control liver. Amino acids in perfusion medium inhibit overall proteolysis and AVs sequestration in both tumor and control hepatocytes, although the inhibition is stronger in control hepatocytes. Thus, even highly neoplastic cells maintain their ability to respond to physiologic regulators.

INTRODUCTION

The major route of hepatocytic protein degradation is known to take place along the lysosomal pathway (Marzella and Glaumann, 1987). Amino acids principally affect only the lysosomal pathway of proteolysis and they have long been known to be important regulators of liver protein degradation (Woodside and Mortimore, 1972; Seglen, 1987). There is strong evidence that amino acid deprivation during liver perfusion increases the number of autophagic vacuoles (AVs) and concomitantly stimulates lysosomally mediated proteolysis in normal hepatocytes (Schworer *et al.*, 1981). On the other hand, addition of high amino acid concentrations to the perfusion medium suppresses protein degradation, inhibits autophagic sequestration, and decreases the volume of the lysosomal apparatus (mainly AVs). Thus, a general relationship seems to exist between changes in protein degradation and changes in volume fractions of lysosomes (Woodside and Mortimore, 1972; Schworer *et al.*, 1981).

It is not known if protein degradation is suppressed by the amino acids them-

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selves, by their metabolites, or by some other effector molecules (Seglen, 1987). The list of amino acids found to be inhibitory in the perfused liver or isolated cells contains leucine, phenylalanine, tyrosine, tryptophane, histidine, methionine, glutamine, and proline (Pösö *et al.*, 1982), of which leucine is the most effective inhibitor. During physiologic growth of cytoplasm, decreased protein degradation seems to be the mean operating mechanism rather than increased protein synthesis (Scornik and Botbol, 1987). As regards proteolysis in rapidly dividing tumor cells which are often characterized by cytoplasmic growth and cell division, our information is still incomplete.

Control of protein degradation has been examined in tumor cells and has been compared with normal cells (Scornik *et al.*, 1978). For example, cancer cell lines exhibit in general lower overall degradation rates than their untransformed counterparts do (Ballard *et al.*, 1980b). In general, however, they show normal sensitivity toward degradation-inhibitory and -stimulatory hormones, growth factors, and serum (Ballard *et al.*, 1980a, b; Cockle and Dean, 1984).

A positive correlation between protein degradation and size of the lysosomal autophagic apparatus for transformed cells also seems to exist (Knecht *et al.*, 1984). We recently reported results indicating that the proteolytic rate is lower in lysosomes from premalignant hepatocyte nodules than in lysosomes from control liver (Ahlberg *et al.*, 1987b).

Since it is difficult to isolate highly purified lysosomes from tumor hepatocytes mainly because of contamination by lysosomes deriving from nontumor cells (Yucel *et al.*, 1988), the perfused liver system of hepatoma-bearing rats was selected in this study for the investigation of proteolysis in tumor tissue. Furthermore, the effect of amino acids and other agents on proteolysis is easier to study *in vitro* than *in vivo,* since fluctuating, often uncontrollable concentrations of amino acids in the blood are avoided. Another advantage with the liver perfusion technique is that it permits the measuring of proteolysis for several hours (Ahlberg *et al.*, 1987a).

The aims of the present study were (i) to examine the proteolysis in malignant hepatocytes (rat hepatoma cells) and investigate the lysosomal responses to amino acid deprivation during perfusion; (ii) to isolate and compare lysosomes (dense bodies and autophagic vacuoles) from tumor and control hepatocytes after perfusion with respect to proteolysis, protein content, and marker enzyme activities; and (iii) to characterize both rat hepatoma cells and isolated lysosomes ultrastructurally and morphometrically.

MATERIALS AND METHODS

Animals and Production of Hepatoma Cells

Nonstarved, male Wistar rats (150–160 g) were used as liver donors. The rats were maintained on stock food and water *ad libitum* in an environmentally regulated room with automatic light control. For the production of hepatocellular cancer the rats were injected ip with diethylnitrosamine, 200 mg/kg body wt. Two weeks later the animals were fed a diet containing 0.02% 2-acetylaminofluorene (2-AAF) for 2 weeks as described by Solt and Farber (1976). In the middle of this period, the animals were two-thirds hepatectomized. Most of the rats developed hepatocellular cancer after 1 year. Control rats were also partially hepatectomized and kept for the same time period.

Perfusion and Subfractionation of Livers

The rats were anesthetized with mebumal (60 mg/ml), 0.1 ml/100 g body wt. Heparin (100 IU in 0.2 ml 0.9% NaCl) was injected into the femoral vein. The abdomen was opened and the portal vein and bile duct were rapidly cannulated with Teflon tubes. The liver was perfused *in situ* and then removed and connected to the perfusion apparatus as detailed previously (Beije *et al.*, 1979). Livers were perfused in a recirculating system with a perfusion medium consisting of Krebs–Ringer bicarbonate solution containing 23% (v/v) washed bovine erythrocytes, 3% (w/v) bovine serum albumin (Sigma, fraction IV), 10 m*M* glucose, 1000 IU heparin, and 2.4 m*M* unlabeled L-leucine to prevent the reutilization of isotope. The pH was adjusted to 7.4 and carbogen gas (O_2 + CO 19:1) was used to oxygenate the perfusate. An amino acid mixture (3 ml) final concentration 1× physiologic levels was added to the perfusate when the purpose was to suppress proteolysis to basal level (Schworer *et al.*, 1981).

At the end of perfusion, usually lasting for 75 min, the liver was flushed with 40 ml 0.3 *M* sucrose to eliminate the perfusate. Control livers and tumor lesions (dissected from the tumor-bearing livers) were then weighed, minced in ice-cold 0.3 *M* sucrose, and homogenized in the same medium with a Potter-Elvehjem glass homogenizer using four complete strokes with a Teflon pestle rotating at 200 rpm.

A lysosomal-enriched fraction was isolated according to the method of Wattiaux *et al.* (1978) with minor modifications (Ahlberg *et al.*, 1982). Lysosomes were collected from the top layer and the 20%/24% Metrizamide interphase. The collected material was pooled, diluted with 0.3 *M* sucrose, and centrifuged at 37,000*g* for 12 min. The resulting pellet was resuspended in 0.3 *M* sucrose (1 g/ml) and used for proteolytic, biochemical, and morphological experiments.

Isotopic Labeling of Proteins and Measurement of Proteolysis

The rats were injected intraperitoneally with L-[1-^{14}C]leucine (CFA 273; 57 Ci/mole), 25 μCi/100 g body wt, 16 hr before sacrifice. This labeling protocol essentially allows measurement only of the degradation of proteins of the slow-turnover pool (long-lived proteins). Samples (0.5 ml) were taken off the perfusate at different time points for measurement of proteolysis. Proteins were precipitated with an equal volume of 5% phosphotungstic acid (PTA) in 2 *M* HCl, and the suspensions were centrifuged at 4000*g* for 20 min. The acid-soluble, isotopically labeled amino acids in the supernatant were measured in an LS 7500 liquid scintillator (Beckman Instruments) after adding 10 ml of Aquasol (New England Nuclear). Proteolysis was defined as release of PTA-soluble dpm/hr in the perfusion fluid and expressed as a percentage of the initial total radioactivity in the liver.

For measurement of protein degradation in homogenate and isolated lysosomes, 1-ml suspensions were incubated at 37°C for 15 and 30 min after adjustment of pH to 5.5 and addition of 5 m*M* DDT (D,L-dithiothreitol, Cleland's reagent). The reaction was stopped by adding an equal volume of 5% PTA, and radioactivity was counted as described above. The results are given as percentage degradation, which denotes PTA-soluble radioactivity divided by total radioactivity in the fraction incubated. For each experiment, a blank was incubated at 4°C and subtracted from the experimental values.

Biochemical Analyses

Protein was determined by the Lowry method (1951) with bovine serum albumin as a standard. Cathepsins B and L were measured as described by Barrett and Kirschke (1981) with Z-arg–arg-NMec (pH 6) and Z-phe–arg-NMec (pH 5.5) as substrates. Acid phosphatase was determined according to the method of Bowers *et al.* (1967). Perfusate samples were taken at different time points for the analysis of LDH (lactate dehydrogenase). Reagents were purchased from Sigma Chemical Co.

Electron Microscopy

Cubes of liver tissue (1 mm^3) were fixed in 3% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.4) containing 0.1 *M* sucrose for 24 hr at +4°C. Aliquots from the fractions were fixed as above for 24 hr. The material was pelleted at 37,000*g* for 60 min. Both liver cubes and fraction samples were rinsed overnight in 0.1 *M* cacodylate buffer (pH 7.4) containing 0.1 *M* sucrose. The material was postfixed in 2% OsO_4 containing 0.1 *M* *s*-collidine buffer (pH 7.4) for 2 hr, dehydrated in a series of graded ethanol solutions and propylene oxide, and embedded in an Epon 812 epoxy resin. Two percent uranyl acetate was added to the final alcohol solution to obtain *en bloc* staining. Ultrathin sections from randomly selected blocks were cut with a diamond knife and stained with lead citrate. Specimens were examined and photographed in an electron microscope (Jeol 100C).

Morphometric Analysis

After calculation of cumulative progressive means, 15–20 randomly obtained electron micrographs at a final standard magnification of ×10,000 were sampled from two blocks from each liver.

The fractional cytoplasmic volume of the lysosomal-autophagic compartment was estimated by the point counting method. Optimal point density was determined as described by Weibel *et al.* (1969). Dense bodies and AVs were identified as described by Pfeifer (1978) using strict morphological criteria. Thus, AVs were defined as a transitional stage between segregation and degradation of cytoplasm. Vacuoles were identified as AVs only if they were demarcated from the cytoplasm by one, two, or in some instances multiple membranes and if they harbored cytoplasmic components (mitochondria, ER, Golgi complex, peroxisomes, or glycogen).

RESULTS

Morphologic Analysis

In this study, malignant rat hepatomas were produced chemically by an injection of diethylnitrosamine followed by the addition of 2-AAF to the food (Solt and Farber, 1976). Partial hepatectomia was performed during feeding with 2-AAF. After some 10–12 months, large liver tumors were obtained. The animals, both control and tumor-bearing rats, were anesthetized and the livers removed and perfused for 75 min. Biopsies were taken from control and tumor livers during perfusion and were fixed for light and electron microscopy. Effects of the addition and deprivation of amino acids on tumor hepatocytes were studied and compared with control hepatocytes.

Light microscopy revealed that the general appearance of the tumor tissue was

a well differentiated hepatocellular carcinoma (not shown). The cells were irregular in shape and size. The ordinary trabecular organization was lost. The nuclei were hyperchromatic with several irregular nucleoli. Frequent cells in mitosis were also present. Principally, two patterns of growth were seen, namely trabecular and glandular lesions. The trabecular carcinoma was composed of thick irregular cords of hepatoma cells, surrounded by numerous small vessels. Tumor cell invasion of vessels was noted. The glandular type of growth consisted of hepatoma cells forming ring structures with a central lumen. A few inflammatory cells and small spotty necroses were also present in the tumor lesions, but not in normal tissue.

Electron microscopy of hepatocytes from control and tumor livers perfused for 75 min without amino acids displayed no apparent ultrastructural changes, other than an increase in the occurrence of AVs (Fig. 1) compared with those perfused with amino acids (Fig. 2).

The ultrastructural appearance of the tumor was that of malignant cells. Mito-

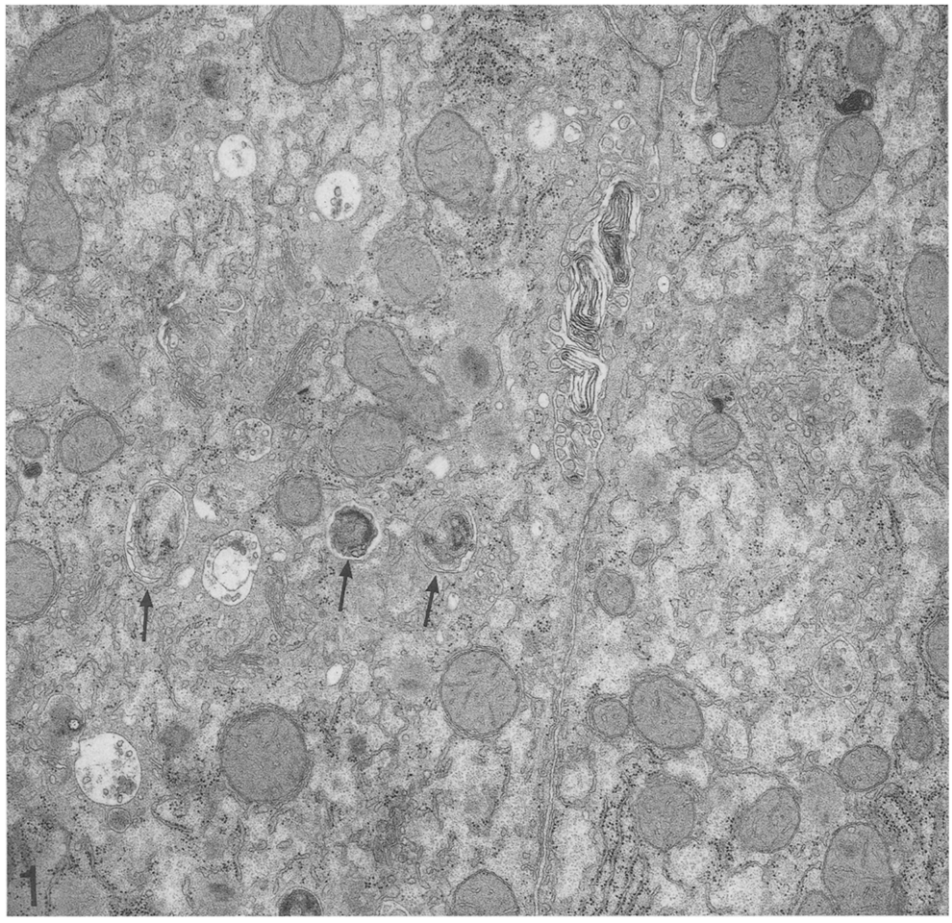

FIG. 1. Ultrastructural appearance of a hepatocyte from a control liver perfused for 1 hr with complete medium except for amino acids. Several autophagic vacuoles (arrows) are seen in various stages of maturation. The picture was chosen from 15–20 randomly obtained electron micrographs sampled from two blocks from each of three livers. Mag. ×14,200.

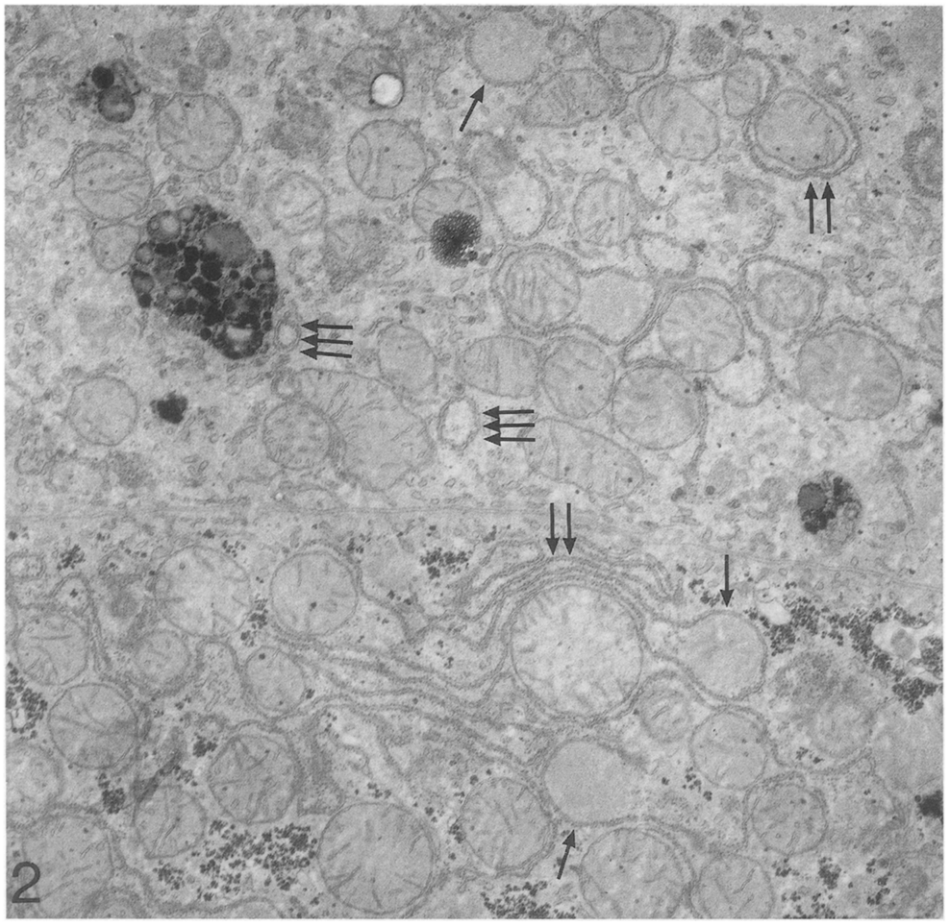

FIG. 2. Ultrastructural appearance of a hepatocyte from a tumor liver perfused 1 hr with complete medium with the addition of amino acids. Note the increased amount of rough-surfaced ER often embracing peroxisomes (single arrows), mitochondria (double arrows), and small portions of cytoplasm (triple arrows), indicating initiated formation of nascent AVs. Mag. ×14,200.

chondria were larger and often irregular compared with their counterparts in nonmalignant tissue. The tumor cell nuclei were irregular and larger. The endoplasmic reticulum (ER), especially rough-surfaced ER, was prominent, often embracing mitochondria, peroxisomes, or small portions of cytoplasm. Bile canaliculi were in general sparse and with clumped microvilli.

The morphometric analysis (*Table I*), performed at the start of and after perfusion, was focused on the lysosomal apparatus, which was divided into its two main components, namely dense bodies and autophagic vacuoles. The components were assessed separately. At the start of perfusion, the cytoplasmic volume of the AVs was several times higher, or 0.35% in hepatoma cells compared with 0.06% in control hepatocytes. Likewise, the volume of dense bodies was somewhat higher, or 0.9% in tumor tissue compared with 0.7% in control tissue (Table I).

The following changes were seen after perfusion:

(i) When the *control livers* were perfused *with* amino acids for approximately 75 min, the

TABLE I

Fractional Cytoplasmic Volume Percent of the Lysosomal Apparatus in Malignant Rat Hepatoma Cells and in Control Hepatocytes with and without the Addition of Amino Acids (A.A) to the Perfusate

	Autophagic vacuoles		Dense Bodies		Total	
Perfusion time (min):	0	75	0	75	0	75
1. Control with A.A	0.059 ± 0.03	0.34 ± 0.04	0.690 ± 0.08	1.98 ± 0.38	0.749 ± 0.10	2.32 ± 0.44
2. Control without A.A	0.063 ± 0.03	1.36 ± 0.20	0.680 ± 0.07	0.55 ± 0.12	0.744 ± 0.09	1.91 ± 0.27
3. Tumor with A.A	0.353 ± 0.05	0.99 ± 0.28	0.940 ± 0.15	1.09 ± 0.30	1.293 ± 0.18	2.08 ± 0.47
4. Tumor without A.A	0.361 ± 0.05	1.58 ± 0.18	0.900 ± 0.15	0.61 ± 0.09	1.261 ± 0.18	2.19 ± 0.23

Note. Tumor and control livers were removed from the rats and perfused for 75 min. Biopsies, taken from livers at the beginning and at the end of perfusion, were fixed immediately in glutaraldehyde and prepared for electron microscopy. Ten to fifteen randomly obtained electron micrographs at a final standard magnification of ×10,000 were sampled from each biopsy. The fractional cytoplasmic volume of the entire lysosomal compartments was estimated by the point counting method as described under Materials and Methods. Means of three experiments ± SE.

cytoplasmic volume of AVs had increased almost six times (from 0.059 to 0.34%) and the volume of dense bodies three times (from 0.69 to 1.98%).

(ii) When *control livers* were perfused *without* amino acids, the cytoplasmic volume of AVs increased 22 times (from 0.063 to 1.36%), whereas that of dense bodies remained almost unchanged.

(iii) When *tumor livers* were perfused *with* amino acids, the cytoplasmic volume of AVs increased three times (from 0.35 to 0.99%) while the volume of dense bodies was unaffected.

(iv) When *tumor livers* were perfused *without* amino acids, the cytoplasmic volume of AVs increased four times (from 0.36 to 1.58%). The volume of dense bodies was somewhat lower, or 0.61% compared with 0.90% at perfusion start.

The following sums up the data presented in Table I: On comparing control and tumor tissue, the relative cytoplasmic volume of AVs was six times higher in tumor tissue at the beginning of perfusion. Furthermore, the results from the morphometric analyses indicate that amino acids inhibit the autophagic sequestration in both tumor and control hepatocytes although the inhibition is stronger in the control cells. The transition of AVs into dense bodies (i.e., degradation of sequestered material) is prolonged in malignant hepatoma cells.

Liver Proteolysis as Estimated from Perfusates and Isolated Lysosomes

To assess the proteolytic rate, control rats and rats bearing liver tumors were injected with [^{14}C]leucine 16 hr before sacrifice. An amino acid mixture at physiologic concentration was added to the recirculating perfusion system when the purpose was to suppress proteolysis close to basal level (Schworer *et al.*, 1981).

Proteolysis as estimated from perfusates without amino acids (Fig. 3) proceeded in control tissue at a rate of 1.73%/hr and in malignant hepatoma at a rate of 0.49%/hr. Such a degradation rate corresponds to a calculated mean half-life ($t_{1/2}$ = 0.693/degradation rate constant) of liver proteins of 40 hr for control and of 141 hr for tumor cells, indicating that tumor cells had at least 50% lower overall protein degradation than normal cells. The actual rate of proteolysis in hepatoma cells is likely to be somewhat lower, since the adjacent nontumor tissue (some 40% of tissue weight) will also add degradation products and thus mask the contribution from the hepatoma cells.

The addition of amino acids to the perfusion medium decreased the release of protein degradation products, compared with depleted medium in both control and tumor liver with degradation rates of 0.78 and 0.33%/hr, respectively. The inhibition of total proteolysis was higher in control hepatocytes (55%) than in

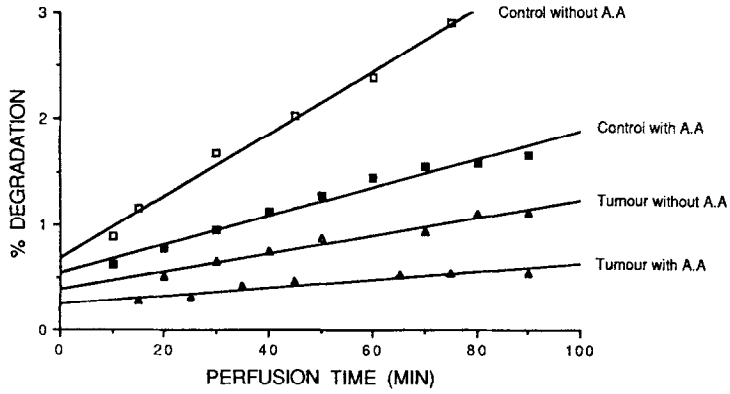

FIG. 3. Lysosomal proteolysis as estimated from perfusates. Livers of rats, injected with L-[1-^{14}C]leucine 16 hr before sacrifice, were perfused for 75 min in a recirculating system *with* and *without* the addition of amino acids to the perfusate. Samples (0.5 ml) were taken off the perfusate at indicated time points. Liver proteolysis was calculated as PTA-soluble radioactivity released during perfusion divided by total initial liver radioactivity. Each point is the mean of three experiments.

tumor hepatocytes (33%). Following the addition of amino acids, estimated half-lives for proteins were 89 hr for control and 210 hr for hepatoma. Accordingly, a lower rate of proteolysis remained in tumor tissue also after addition of amino acids.

As detailed earlier, isolated lysosomes from malignant rat hepatoma cells have lower proteolysis than control lysosomes (Yucel *et al.*, 1988). Lysosomes isolated from a control liver perfused *with* amino acids had 46% lower proteolysis than proteolysis in lysosomes isolated from a control liver which had been perfused *without* amino acids (Fig. 4). Interestingly, lysosomes isolated from tumor liver tissue perfused *with* amino acids (Fig. 5) displayed only 25% lower proteolysis than that of lysosomes isolated from a tumor liver tissue perfused *without* amino

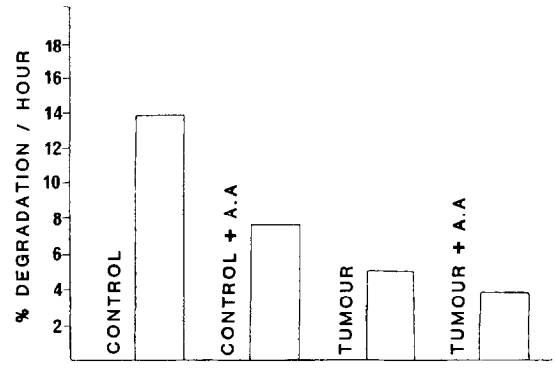

FIG. 4. Proteolysis in isolated lysosomes from malignant and control liver tissue. Rats were injected with L-[1-^{14}C]leucine 16 hr before sacrifice and the livers were removed and perfused *with* and *without* amino acids. The lysosomal fractions were prepared as described under Materials and Methods and suspended in 0.3 *M* sucrose (approximately from 1 g liver/ml). One-milliliter suspensions were incubated for 15 and 30 min and the reaction was stopped by adding an equal amount of 5% PTA. The proteolysis was measured as PTA-soluble activity in the supernatant after centrifuging at 4000*g* for 20 min. Percentage degradation denotes PTA-soluble radioactivity divided by total radioactivity in the fraction. Means of three experiments.

acids. Thus, in this case also, malignant hepatocytes responded in a similar fashion, although to a lesser extent than normal liver.

Biochemical Analyses

Protein content and lysosomal enzymes were measured in homogenate and lysosomal fractions. The protein contents (mg/g of liver) of homogenate and of lysosomes were somewhat lower in tumor than in control tissue (Table II). The activities (per gram of liver) of the lysosomal enzymes acid phosphatase, and the two principal proteinases, cathepsin B and cathepsin L, were 50% lower in homogenate and some 75–85% lower in the lysosomal fraction from tumor hepatocytes than in the corresponding fractions from control liver.

LDH activities were measured continuously in the perfusion medium, as a marker enzyme for liver cell lysis (not shown). The leakage of LDH from the liver cells was negligible as estimated by the proportion of LDH in the perfusate, which increased from only 0.1 to 0.2% in control and from 0.1 to 0.3% in tumor livers. (Percentage denotes activity in the whole perfusion medium divided by total activity in the liver.)

DISCUSSION

The lysosomal apparatus has been shown to be the paramount locus of protein degradation in normal rat liver (Ahlberg *et al.*, 1985; Marzella and Glaumann, 1987; Mortimore, 1987), whereas little is known as regards its role in malignant cells (Ahlberg *et al.*, 1987b; Knecht *et al.*, 1984). It has been suggested that the increase in size and growth of cells could occur as a result of a reduction in intracellular protein degradation (Scornik *et al.*, 1978) rather than from increased protein synthesis. In the present study, we performed ultrastructural analysis of and measured total and lysosomal protein degradation in the perfused, chemically induced rat hepatoma liver. The perfused rat liver system was chosen to be able to assess total proteolysis in this organ and to avoid the effects of hormones and other substances at unknown concentrations in the blood (Ahlberg *et al.*, 1987a). More precisely, the purpose was to assess earlier *in vitro* studies indicating lower proteolysis in tumor cells and evaluate the importance of amino acids for the regulation of lysosomal proteolysis in rat hepatoma cells.

Light and electron microscopical studies revealed that the tumor tissue was a

TABLE II
Protein and Selected Enzyme Activities in Liver Homogenate and Lysosomal Fractions

	Fraction	Protein (mg/g)	Acid phosphatase[a] (act/g)	Cathepsin B[b] (μM/g)	Cathepsin L[b] (μM/g)
1. Control with A.A	Homogenate	231	638	613	9500
	Lysosomes	1.04	48	40	1195
2. Control without A.A	Homogenate	219	570	480	19600
	Lysosomes	1.0	46	40	1326
3. Tumor with A.A	Homogenate	197	370	288	4625
	Lysosomes	0.71	7.4	5.4	173
4. Tumor without A.A	Homogenate	136	313	240	3963
	Lysosomes	0.61	7.5	7.5	361

Note. The homogenate and lysosomal fractions were prepared after perfusing the livers with and without A.A (amino acids) as described under Materials and Methods. Means of three experiments.

[a] Micromoles of phosphate released per minute per gram of liver.

[b] Micromoles of NMec released per minute per gram of liver.

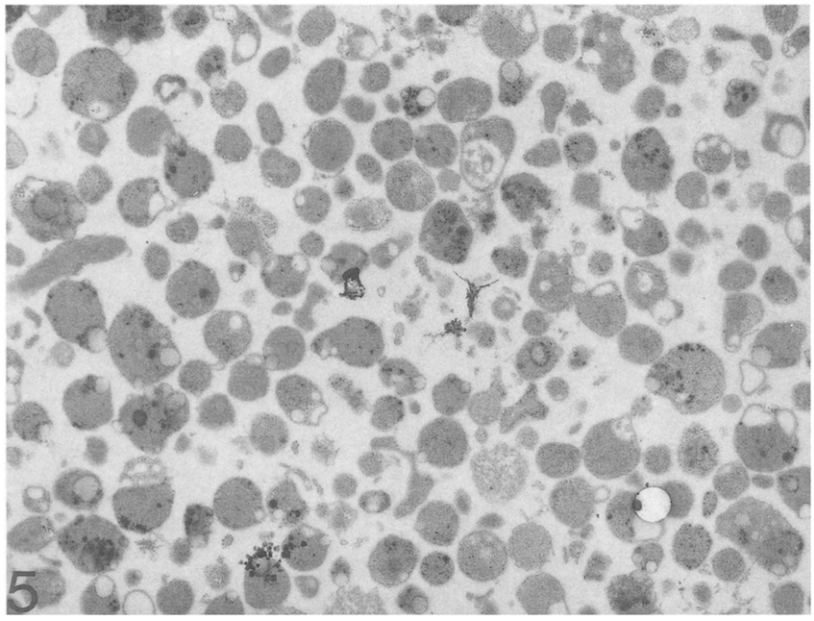

FIG. 5. Ultrastructural appearance of lysosomes isolated from malignant liver tissue. Chemically induced rat hepatoma was perfused for approximately 75 min with amino acids in the medium. Lysosomes were then isolated and prepared for electron microscopy as described under Materials and Methods. The fraction consists of a population of dense bodies with only a few contaminating nonlysosomal organelles. Mag. ×8,800.

well-differentiated hepatocellular carcinoma. Fractional cytoplasmic volume of the lysosomal apparatus (dense bodies and autophagic vacuoles) based on electron microscopy was obtained for hepatoma cells and control hepatocytes which had been perfused *with* and *without* an amino acid mixture. Compared with control hepatocytes, tumor hepatocytes had a larger cytoplasmic volume of AVs at the beginning of perfusion. This may have several explanations. One could be that AVs in tumor hepatocytes are formed at a rate similar to that in normal liver cells but exhibit a longer life span.

Decreased synthesis and ensuing lower activities of lysosomal proteolytic enzymes as found here would result in a longer destruction time for sequestered material and accordingly increase the half-life of AVs. Another possibility might be that the fusion of nascent autophagosomes with primary or secondary lysosomes becomes altered or diminished by some unknown mechanism, e.g., disturbed organelle traffic. This would also result in lower enzyme activities in secondary lysosomes. Our data are commensurate with the notion that the decrease in lysosomal enzyme activities could cause a prolongation of the transition of AVs to dense bodies and thus also of their life span. This, in turn, would result in an increased occurrence of AVs in transition to dense bodies, as also found in our morphometric analysis. The increased numbers of AVs and dense bodies seen after perfusion even in the presence of amino acids are at variance with our previous studies in which the lysosomal apparatus remained unchanged during

perfusion (Henell and Glaumann, 1985). However, in the earlier experiments we used 4× physiologic concentrations of amino acids. In the present study normal serum concentrations of amino acids were preferred. The principal difference in AV volume between control and tumor tissue also remained when 4× physiologic concentration was used (not shown).

It has been demonstrated that high amino acid concentrations in perfusion medium suppress protein degradation and inhibit autophagic sequestration in normal cells (Woodside and Mortimore, 1972; Schworer *et al.*, 1981). Our studies reveal that amino acids in the perfusion medium inhibit autophagic sequestration by 77% in control hepatocytes as against 48% in malignant hepatocytes. The lower inhibition of the formation of AVs by amino acids in tumor cells could be due to an intrinsically low autophagic sequestration rate in malignant liver cells that is difficult to further inhibit. This would indicate that tumor cells are less dependent on amino acids, which is in line with findings by Schwarze and Seglen (1985) who observed that preneoplastic hepatocytes survived in an amino-acid-free culture medium, whereas normal hepatocytes died rapidly. In line with a higher autonomy of tumor cells, we recently reported that malignant hepatoma cells are also less responsive to leupeptin (Yucel *et al.*, 1988).

When protein degradation was assessed by perfusing livers containing isotopically prelabeled proteins, different proteolytic rates were encountered for tumor and control livers. The total proteolysis was 50% lower in malignant cells than in control cells. Similarly, isolated lysosomes from hepatoma cells (Fig. 5) displayed lower proteolysis than control cells. Accordingly, there is positive correlation between decreased overall protein degradation as assessed in the perfusate and decreased proteolysis as assessed in isolated lysosomes. This strongly indicates that the regulation of protein degradation is principally modulated by the proteolytical activity of the lysosomal apparatus. This also holds for normal hepatocytes.

The lower degradation rates of cellular proteins and relative insensitiveness to amino acids in hepatoma cells might give them an advantage for faster growth over their neighboring normal hepatocytes.

The conclusions of our work are that the cytoplasmic volume of AVs is larger and that both the overall and the lysosomal proteolysis is lower in tumor liver than in control liver. Lysosomes from malignant hepatoma cells display only a fraction of principal proteolytic enzyme activities. This might explain the large cytoplasmic volume of AVs in hepatoma cells. Amino acids in the perfusion medium inhibit overall proteolysis and autophagic sequestration in both tumor and control hepatocytes, although the response is less in the malignant cells.

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