Early development of protein metabolic perturbations in the liver and skeletal muscle of tumour-bearing rats

A model system for cancer cachexia

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In rats into which a fast-growing ascites hepatoma (Yoshida AH-130) had been transplanted, tumour growth elicited a marked loss of body weight until the animal's death in about 2 weeks. Overall tissue protein metabolism was simultaneously studied *in vivo* in the gastrocnemius muscle and liver after labelling with [^{14}C]bicarbonate. Early and progressive atrophy developed in the gastrocnemius muscle, the underlying metabolic imbalance being expressed by an elevation in the apparent protein-degradation rate, with no changes in the apparent synthesis rate. A transient hyperplastic response preceded waste in the liver, both states being associated with alterations in protein-degradation rate: an initial decrease during liver growth, then an acceleration as liver regressed. Protein-synthesis rates, virtually unchanged during liver growth, were elevated in the subsequent phase, although not sufficient to balance the enhanced breakdown. Thus, in the tumour host tissues examined, altered states of protein turnover appeared to result mostly from changes in rates of protein breakdown. In sharp contrast with the negative protein balance in the host, the ascites hepatoma cells had the ability to grow or at least, in advanced stages, to maintain a stationary state.

INTRODUCTION

Malignancies may engender complex metabolic disturbances in both humans and experimental animals, resulting in rapid body-weight loss and tissue wasting. Atrophy is particularly striking in skeletal muscle and adipose mass, whereas organs such as liver, kidneys, adrenals or spleen may actually enlarge, transiently at least (see Lawson *et al.*, 1982).

The skeletal muscle, which embodies the major protein mass of the organism, is severely affected in cancer cachexia. Protein waste implies an imbalance between synthesis and degradation; whether the primary effect is on protein synthesis or protein degradation remains debatable, in view of the many conflicting reports in the literature (see the Discussion section). Different primary or transplanted tumours may cause transient liver growth, largely hyperplasia (Morgan & Cameron, 1973); liver wasting, however, eventually follows. Again, there is no agreement as to whether this liver wasting is secondary to a decrease in protein synthesis or an increase in protein breakdown (see the Discussion section).

To investigate how protein metabolism changes in cancer host tissues, we transplanted a highly deviated fast-growing ascites hepatoma (Yoshida AH-130) into rats. We have previously reported studies on the changes occurring in protein turnover in these tumour cells during growth-phase transitions (Baccino *et al.*, 1984, 1985). The body weight decreases early in rats into which this tumour has been transplanted, and early changes develop in liver and gastrocnemius muscle (Tessitore *et al.*, 1985). Whereas most previous investigations have dealt with fully established cachexia (e.g. Emery *et al.*, 1984a,b; Pain *et al.*, 1984), the present work was designed to analyse the series of events that ultimately develop into cachexia. For this purpose, overall protein metabolism has been simultaneously

monitored in both rat liver and gastrocnemius muscle after transplantation with the AH-130 hepatoma. Additionally, these data have been evaluated in relation to the growth phase, size and protein-turnover state of the tumour itself.

MATERIALS AND METHODS

Chemicals

$\text{NaH}^{14}\text{CO}_3$ (53 mCi/mmol) was from New England Nuclear, Boston, MA, U.S.A., Picofluor 30 from Packard Instruments International, Zurich, Switzerland, and crystallized bovine serum albumin from Sigma Chemical Co., St. Louis, MO, U.S.A.

Animals

Male Wistar rats (Nossan, Milano, Italy) weighed about 200 g; they were maintained on a regular light–dark cycle (light 08:00–20:00 h) and had free access to a balanced diet (Piccioni, Brescia, Italy). Rats were injected intraperitoneally with $\text{NaH}^{14}\text{CO}_3$ (400 $\mu\text{Ci/kg}$ body wt.) dissolved in 0.15 M-NaCl, 24 h before tumour transplantation. At zero time animals were randomized into two groups: the first received an intraperitoneal inoculum of 10^8 Yoshida ascites-hepatoma cells obtained from exponentially growing tumours; the second one was untreated and served as control. At zero time and every second day at 10:00 h all rats were weighed, and six per group were killed by decapitation. Tumour volume, cellularity and wet weight were evaluated; liver and gastrocnemius muscle were rapidly weighed and homogenized (see Baccino *et al.*, 1982).

Assays

Protein was determined by the method of Hartree (1972) and DNA by the method of Fiszer-Szafarz *et al.* (1981), the standards being bovine serum albumin and

calf thymus DNA respectively. Trichloroacetic acid-insoluble proteins were processed for lipid extraction (Swick & Ip, 1974), extensively hydrolysed (12 h at 90 °C) in 0.3 M-NaOH, and counted for radioactivity by liquid scintillation (for further details, see Baccino *et al.*, 1982).

Fractional protein turnover rates

Total protein content, total protein radioactivity and specific protein radioactivity (radioactivity per mg of protein) were evaluated for liver and gastrocnemius at each time point. Fractional rates of protein synthesis (k_s), degradation (k_d), and accumulation (k_a , k_a') were calculated by the following equations:

$$k_s = \ln(\text{specific protein radioactivity})/t$$

$$k_d = \ln(\text{total protein radioactivity})/t$$

$$k_a = \ln(\text{total protein})/t$$

$$k_a' = k_s - k_d$$

as previously reported (Amenta *et al.*, 1978; Baccino *et al.*, 1982) and expressed as %/h. Apparent half-lives ($t_{1/2}$) are given in days. The procedure adopted in studying protein radioactivity decay, with zero time corresponding to 24 h after administration of the label, only takes into account the long-lived pool of tissue proteins, which represents the large majority of the protein mass (cf. Amenta *et al.*, 1980).

RESULTS

Effects of tumour growth on body and tissue weight

With an inoculum of 10^8 Yoshida AH-130 cells, the tumour population increased exponentially in size for

about 6 days, with a doubling time of 1 day; then growth slowed and the tumour eventually shifted into a quasi-stationary state on day 8 (Table 1). The ascites hepatoma was uniformly lethal within 14–16 days after transplantation.

The rat body weight, exclusive of the tumour, progressively declined in tumour hosts down to 50% of control values by day 10, the end of the present experimental period; by contrast, it regularly increased in controls (Table 1). For purposes of standardization, all data on muscle and liver were thus referred to 100 g of initial body wt. (cf. Baccino *et al.*, 1982).

In tumour-transplanted animals (Table 1), gastrocnemius muscles showed a progressive decrease in wet weight and total protein after day 2; by day 10 these values were 37–40% of the comparable 10-day controls. In contrast, liver changes followed a bimodal pattern: first a moderate increase in wet weight and total protein content was observed (10–15% by day 4), corresponding to the exponential phase of tumour growth; then both parameters showed a slight decrease, reaching 80–85% of comparable control values by day 10 (or less, in other experiments). Changes in body weight and liver were already evident 2 days after tumour transplantation, whereas those affecting the gastrocnemius muscle developed slightly later (Fig. 1). AH-130 cells amounted to about 250 mg wet wt. after 2 days of growth, providing a tumour burden of not more than 0.12% of host body weight; by day 4 the tumour burden still was no more than 0.5% of host body weight.

Protein turnover in liver and skeletal muscle

A single dose of $\text{NaH}^{14}\text{CO}_3$ was administered to rats 24 h before tumour transplantation (zero time); total and specific radioactivity of tissue protein as well as total

Table 1. Body weights, tissue weights and protein content in control and AH-130-tumour-bearing rats

Tissue weight, total protein and DNA are expressed per 100 g initial body wt. Values for host body wt. are exclusive of tumours. Values for gastrocnemius refer to a single muscle, averaging data for the two muscles in each animal. Tumour AH-130 cellularity, protein and DNA are expressed per total tumour. Results are means $\pm$ S.D. ($n = 6$).

	Time after tumour transplantation (days)					
	0	2	4	6	8	10
Controls						
Body wt. (g)	199 $\pm$ 19	209 $\pm$ 20	222 $\pm$ 9	225 $\pm$ 18	235 $\pm$ 16	260 $\pm$ 25
Muscle wet wt. (mg)	500 $\pm$ 32	523 $\pm$ 37	608 $\pm$ 19	633 $\pm$ 60	697 $\pm$ 32	783 $\pm$ 49
Muscle protein (mg)	104 $\pm$ 11	110 $\pm$ 6	133 $\pm$ 14	136 $\pm$ 12	170 $\pm$ 7	171 $\pm$ 20
Liver wet wt. (g)	4.4 $\pm$ 0.2	4.6 $\pm$ 0.1	5.0 $\pm$ 0.5	5.1 $\pm$ 0.4	5.4 $\pm$ 0.5	5.8 $\pm$ 0.6
Liver protein (mg)	1276 $\pm$ 106	1412 $\pm$ 93	1517 $\pm$ 100	1445 $\pm$ 139	1616 $\pm$ 252	1650 $\pm$ 257
Liver DNA (mg)	12.0 $\pm$ 1.4	13.4 $\pm$ 0.6	13.3 $\pm$ 1.9	12.8 $\pm$ 0.5	13.6 $\pm$ 1.2	13.6 $\pm$ 2.1
Tumour hosts						
Body wt. (g)	—	195 $\pm$ 22	176 $\pm$ 14	160 $\pm$ 22	140 $\pm$ 22	130 $\pm$ 14
Muscle wet wt. (mg)	—	523 $\pm$ 32	495 $\pm$ 41	407 $\pm$ 50	358 $\pm$ 49	310 $\pm$ 42
Muscle protein (mg)	—	107 $\pm$ 15	103 $\pm$ 15	75 $\pm$ 17	76 $\pm$ 13	63 $\pm$ 14
Liver wet wt. (g)	—	5.1 $\pm$ 0.3	5.5 $\pm$ 0.5	5.3 $\pm$ 0.4	5.1 $\pm$ 0.6	4.6 $\pm$ 0.3
Liver protein (mg)	—	1526 $\pm$ 116	1740 $\pm$ 208	1563 $\pm$ 71	1497 $\pm$ 292	1406 $\pm$ 121
Liver DNA (mg)	—	15.1 $\pm$ 2.3	17.9 $\pm$ 3.3	15.8 $\pm$ 1.9	15.4 $\pm$ 0.9	14.1 $\pm$ 1.1
AH-130 tumours						
Volume (ml)	—	3.3 $\pm$ 1.2	13.5 $\pm$ 4.7	30.5 $\pm$ 10.0	58.6 $\pm$ 21.0	85.4 $\pm$ 28.0
Cell number ($\times 10^7$)	—	16 $\pm$ 3	76 $\pm$ 22	220 $\pm$ 83	414 $\pm$ 99	438 $\pm$ 76
Cell protein (mg)	—	61 $\pm$ 26	234 $\pm$ 71	980 $\pm$ 53	1613 $\pm$ 49	1351 $\pm$ 42
Cell DNA (mg)	—	0.9 $\pm$ 0.4	5.1 $\pm$ 2.0	14.6 $\pm$ 6.0	21.9 $\pm$ 5.7	24.7 $\pm$ 7.1

Fig. 1. Body (○), liver (△) and gastrocnemius (□) weights compared with AH-130 tumour growth (●) in rats

Body and tissue wet weights in tumour bearers are expressed as percentages of control values (see Table 1). Vertical bars denote S.D. ($n = 6$).

protein content were then measured at zero time and every second day thereafter (Figs. 2 and 3). The 24 h interval between tracer administration and the first measurement was sufficient to eliminate any fast-turnover component of protein turnover; this experimental protocol thus measured only protein in the slow-turnover pool and excluded secretory proteins. It should be noted that $\text{NaH}^{14}\text{CO}_3$ was used as a single radioactive precursor for liver and gastrocnemius protein; it is well known that such a labelling procedure, although most suitable for liver protein (Swick & Ip, 1974), suffers from considerable label recycling in skeletal muscle (MacDonald *et al.*, 1979). Although methods are available to obtain reliable estimates of protein-synthesis rates in muscle (Garlick *et al.*, 1980), the procedure adopted here seemed to offer the best compromise to monitor simultaneously protein-synthesis and degradation rates for liver and muscles in the same animals.

In 10 days, the percentage loss of total protein radioactivity from gastrocnemius muscles was 37% in controls and 76% in experimental rats (Fig. 2). Over the entire experimental period, the decay of total protein radioactivity in gastrocnemius muscles fitted quite closely a first-order rate in both groups; however, the apparent k_d remained consistently higher in tumour hosts (0.62%/h) than in controls (0.20%/h). Over the same period, the specific radioactivity of gastrocnemius protein decayed at quite similar rates for tumour-bearing and for control animals. The present estimates of apparent k_s and k_d for control muscle protein (see Table

Fig. 2. Decay of specific (a) and total (b) protein radioactivity in gastrocnemius muscles of control (△, ○) and AH-130-tumour-bearing (▲, ●) rats

Each point represents the mean for six animals (S.D./mean $\leq 10\%$). Values for apparent k_s and k_d , calculated by regression analysis, are reported in Table 2.

2) compare well with data in the literature, obtained through different approaches (see e.g., Stein *et al.*, 1976; Waterlow *et al.*, 1978; Millward, 1980; Bates & Millward, 1983; Pain *et al.*, 1984). Constancy of the apparent k_s and k_d over the 10 days experimental period suggests that in gastrocnemius muscles these parameters mostly measured a single large protein pool, the turnover of which was definitely accelerated in tumour bearers (apparent $t_{1/2}$ of 4.7 days, versus 14.4 days in controls). Of interest in this connection is the observation that muscular protein depletion in tumour hosts mostly involves the myofibrillar system, particularly in muscles such as the gastrocnemius (Clark & Goodlad, 1971); in normal rats, the half-lives of muscular contractile proteins are of the order of a few weeks (Waterlow *et al.*, 1978; Bates & Millward, 1983).

Unlike in gastrocnemius muscles, the decay of specific or total protein radioactivity in control livers (Fig. 3) did not fit a simple exponential curve, probably owing to the increasing prevalence of components of progressively longer half-lives in the total protein pool in the liver (cf. Swick & Ip, 1974; Garlick *et al.*, 1975). Therefore, fractional rates of protein synthesis or degradation could

Table 2. Metabolic parameters for protein of the slow-turnover pool in liver, gastrocnemius and AH-130 tumour cells

The 'Time (days)' column refers to the time interval after tumour transplantation. Fractional rates are expressed as %/h; $t_{1/2}$ is given in days. Note that the time interval for measurements on exponential or stationary tumour cells was from day 1 to day 3 after labelling, whereas it was from day 3 to day 11 for liver and muscles. In principle, such difference should have resulted in underestimating both k_s and k_d for tissues in the time interval corresponding to the stationary phase of tumour growth. This was indeed the case for control livers, and possibly also for tumour-host livers; yet for muscle k_s and k_d in the two time intervals did not differ appreciably.

	Time (days)	k_s	k_d	$t_{1/2}$	k_a'	k_a
Liver						
Controls	0-4	1.11	0.93	3.11	0.18	0.18
	6-10	0.64	0.58	4.98	0.05	0.13
AH-130 hosts	0-4	1.18	0.79	3.66	0.39	0.32
	6-10	0.99	1.14	2.53	-0.15	-0.11
Muscle						
Controls	0-4	0.46	0.20	14.44	0.26	0.23
	6-10	0.46	0.20	14.44	0.26	0.23
AH-130 hosts	2-4	0.40	0.62	4.66	-0.22	-0.23
	6-10	0.40	0.62	4.66	-0.22	-0.23
AH-130 cells						
Exponential	2-4	3.10	0.61	4.73	2.49	2.77
Stationary	9-11	1.49	1.43	2.02	0.06	0.00

not be calculated over the whole experimental period. For practical purposes, this was conveniently split into two intervals, days 0-4 and days 6-10 respectively,

Fig. 3. Decay of specific (Δ , $\triangle$) and total ($\bullet$, $\circ$) protein radioactivity in the liver of control (Δ , $\circ$) and AH-130-tumour-bearing ($\bullet$, $\triangle$) rats

Each point represents the mean from six animals (S.D./mean $\leq 10\%$). Values for apparent k_s and k_d , calculated by regression analysis, are reported in Table 2.

corresponding roughly to the periods of rapid and stationary tumour growth. Comparisons between controls and tumour hosts were thus established in these two time ranges (see Table 2). In tumour-bearing rats, the initial net accumulation of liver protein was associated with a moderate decline of k_d , without any significant change of k_s , whereas the subsequent phase of protein loss was characterized by an elevation of k_s and, more marked, of k_d .

Protein turnover in host tissues compared with tumour cells

The protein metabolism parameters for liver and gastrocnemius muscle in controls and tumour bearers are summarized in Table 2. The rates for protein accumulation (or loss) calculated by difference between synthesis and degradation rates ($k_a' = k_s - k_d$) matched those determined directly from total protein data (k_a), thus checking the internal consistency of the data.

For purposes of comparison, Table 2 also includes similar parameters previously measured on AH-130 tumour cells (Baccino *et al.*, 1984, 1985). Data in Table 2 thus clearly show that, whereas tumour cells exhibited pronounced alterations in both k_s and k_d on cessation of growth, the major changes in host liver and muscle always involved k_d . Moreover, the neoplastic population still maintained a balance between synthesis and degradation in the stationary phase, in spite of a marked elevation of k_d , whereas at any time elevation of k_d in host tissues was associated with decrease in the protein mass.

DISCUSSION

A model system for cancer cachexia has been delineated in the present work. In addition to the simplicity of the technique, other advantages were: (1) the prompt development of perturbations in hosts and

host tissues after tumour implantation; (2) the simultaneous comparison of host and tumour cell metabolism. Moreover, body weight and tissue protein content were already affected when the AH-130 tumour burden amounted to 0.1–0.2% of body weight, which is quite low if compared with that for current model tumours (see e.g., Morrison *et al.*, 1984; Pain *et al.*, 1984; Warren *et al.*, 1985).

Muscle changes

Many conflicting reports have appeared as to the nature of the metabolic alterations responsible for muscle protein waste associated with human or experimental cancer: data support enhanced protein breakdown (Goodlad & Clark, 1973; Goodlad *et al.*, 1981; Elia *et al.*, 1981; Heber *et al.*, 1982), decreased synthesis (Clark & Goodlad, 1971; Stein *et al.*, 1976; Lundholm *et al.*, 1978, 1981, 1982; Bennegård *et al.*, 1982, 1984; De Wys, 1982; Rennie *et al.*, 1983; Emery *et al.*, 1984a,b; Pain *et al.*, 1984), or both (Lundholm *et al.*, 1976; Emery *et al.*, 1982; Kawamura *et al.*, 1982). However, the situations investigated were quite heterogeneous with respect to tumour types, tumour–host relationships, and also the skeletal muscles examined (Goodlad & Clark, 1973; Goodlad *et al.*, 1981).

In the present work, gastrocnemius-muscle atrophy started to develop after the second day after tumour transplantation and was then rapidly progressive. Limited physical exercise or decreased food intake and/or adsorption could contribute to muscle waste in tumour cachexia (Theologides, 1979; Lundholm *et al.*, 1981; Stein, 1982; Emery *et al.*, 1984b; Pain *et al.*, 1984; Waterlow, 1984). Yet animals appeared to maintain a normal locomotive activity for several days after tumour transplantation. In addition, the effects of complete starvation or protein deprivation on muscle protein content are more delayed and less severe than those herewith reported (cf. Garlick *et al.*, 1975; Goodman & Ruderman, 1980).

The present data suggest that in rats bearing tumour AH-130 the imbalance between protein synthesis and degradation mostly resulted from enhanced breakdown. Yet in muscles the label used ($\text{NaH}^{14}\text{CO}_3$) may show considerable recycling (MacDonald *et al.*, 1979); this could have been decreased in tumour hosts, resulting in enhanced efflux of labelled amino acids from the tissue. In this case we would expect an equal effect on the apparent synthesis rate calculated from the change in specific protein radioactivity; the synthesis rate would then be underestimated in controls as compared with tumour-bearing animals. However, the apparent synthesis rates of muscle protein were virtually the same for both experimental groups. Thus an alternative consistent with our results is that a recycling block co-existed with a partial block in protein synthesis; studies by Pain *et al.* (1984) have indeed demonstrated that muscle protein synthesis can be inhibited in tumour-bearing animals.

Liver changes

Tissue protein degradation is markedly slowed down in situations of liver growth (Conde & Scornik, 1976, 1977; Scornik & Botbol, 1976). By contrast, when the hepatic protein mass falls during starvation (Garlick *et al.*, 1975; Goodman & Ruderman, 1980; Baccino *et al.*, 1982) or protein deficiency (Conde & Scornik, 1976;

Goodman & Ruderman, 1980), such loss in the 'early starvation phase' is largely due to enhanced degradation (Khairallah, 1978; Baccino *et al.*, 1982).

The initial liver growth in animals into which a variety of rapidly growing tumours has been transplanted largely reflects hyperplasia (Morgan & Cameron, 1973). The hepatic protein mass likewise increased in animals bearing tumour AH-130; this observation militates against a significant decrease in food intake or absorption during the first days after tumour transplantation. The 'divergent behaviour of liver and muscle' (Lawson *et al.*, 1982) was transient, however, and eventually both tissues showed a marked regression.

Both the initial enlargement and subsequent atrophy of the liver in tumour AH-130 hosts were associated with changes in the protein-degradation rate, this being first decreased and then augmented, respectively. Altered synthesis rates did not appear to contribute substantially to liver changes; surprisingly, synthesis was enhanced in the phase of liver regression, although not enough to compensate for the increased breakdown. Liver growth in rats bearing the Walker 256 carcinoma has also been shown to result from a decrease in protein degradation, with no change in synthesis (Goodlad & Clark, 1973); yet enhanced protein synthesis has been suggested for other model systems (Stein *et al.*, 1976; Lundholm *et al.*, 1978; Clark & Goodlad, 1981; Pain *et al.*, 1984; Emery *et al.*, 1984a). However, in many investigations the methodology was not adequate; particularly, no distinction was made between 'fixed' and secretory liver proteins (cf. Pain *et al.*, 1984; Warren *et al.*, 1985). Overall synthesis of 'fixed' liver proteins in tumour-bearing rats has been found to be either unchanged (Kawamura *et al.*, 1982; De Wys, 1982; Emery *et al.*, 1984a) or even increased, but only for high tumour burdens (Warren *et al.*, 1985). The present observations, based on a method that only measured 'fixed' slow-turnover liver proteins, clearly indicate that duration of tumour growth may be a further important source of variation in such measurements.

Concluding remarks

It has been suggested that the systemic effects of tumours on the host are mediated by circulating factors (Lucké *et al.*, 1952–53), such as altered concentrations of normal hormones as well as other molecules derived from tumour or host cells (Lucké *et al.*, 1952–53; Suda *et al.*, 1966; Parry & Ghadially, 1970; Morgan & Cameron, 1973; Parry, 1976; Shapot, 1979; Lawson *et al.*, 1982; Beutler & Cerami, 1986). Muscle hypotrophy and liver enlargement both develop in rats treated with glucocorticoids (Lunn *et al.*, 1976; Odedra *et al.*, 1983) or endotoxin (Jepson *et al.*, 1986). Alterations in the activity, amount and molecular forms of lysosomal cathepsins, detected in both liver and muscle of AH-130 tumour hosts (Tessitore *et al.*, 1982, 1986a,b; L. Tessitore, G. Bonelli & F. M. Baccino, unpublished work), might also be relevant in this regard (cf. Lundholm *et al.*, 1980; Greenbaum & Sutherland, 1983).

So far, analysis of the present model suggests that protein metabolic alterations in the liver and gastrocnemius muscles of AH-130-tumour-bearing rats mostly reflected catabolic modulations. It cannot be excluded, however, that the observed alterations of the apparent protein turnover rates in host tissues could also reflect, to some extent, changes in radioactive-tracer recycling,

both internal and external (Waterlow *et al.*, 1978), particularly in muscle (MacDonald *et al.*, 1979).

The progressive host tissue waste was in marked contrast with the behaviour of the AH-130 cells; clearly, the latter had the ability to grow, or at least to maintain a stationary state (Baccino *et al.*, 1984; Tessitore *et al.*, 1986a), even when protein metabolism was definitely unbalanced in the host, particularly in some tissues. Data in Table 2 suggest that tumour cells may have a definite selective advantage, particularly on the catabolic side of protein turnover (cf. Baccino *et al.*, 1984; Schwarze & Seglen, 1985; Tessitore *et al.*, 1986a). On the other hand, protein metabolic perturbations in the host, although causing tissue waste, may themselves favour growth of the tumour itself.

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