Protein Synthesis Measured in Vivo in Muscle and Liver of Cachectic Tumor-bearing Mice

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ABSTRACT

Protein synthesis has been measured in vivo in liver and muscle of mice bearing the XK1 tumor, an appropriate model for cancer cachexia. Two different methods were used involving measurement of tracer incorporation into tissue protein either at the end of a 4-hr constant infusion of [14C]tyrosine or 10 min after i.v. injection of a flooding dose of [3H]phenylalanine.

Whole-body tyrosine flux was decreased by 60% in cachectic tumor-bearing mice, and protein synthesis was depressed by 70% in muscle and by 40% in liver. The depression of protein synthesis in muscle was due to a reduction in both RNA content (i.e., protein-synthesizing capacity) and RNA activity (i.e., protein synthesized per g of RNA per hr). In liver, the depression of protein synthesis was due entirely to a decrease in RNA activity. The results also suggest that the synthesis of export proteins was affected more than the synthesis of fixed liver protein.

Restriction of food intake in normal mice by up to 50% caused a loss of body weight and reductions in protein synthesis in liver and muscle which were less severe than those caused by the presence of the tumor. It is concluded that the wasting which is associated with advanced malignant disease is brought about by a reduction in the rate of protein synthesis in the tissues, and that this cannot be explained by depression of food intake alone.

INTRODUCTION

A prominent feature of cancer cachexia is the loss of body protein, but it is not clear whether this is due to changes in the rate of protein synthesis or the rate of protein breakdown, or both. Investigations of this problem using experimental animals have suffered from 2 major methodological limitations. Experimentally induced tumors in animals may comprise more than 30% of the host's body weight, whereas the tumor burden in humans rarely exceeds 5% of body weight (5, 30); tumor implantation may cause a 50% decrease in food intake in experimental animals (15), but the food intake of cachectic cancer patients is probably not reduced by more than 15% (2). Thus, most of the tumor-bearing animals used previously have probably been inappropriate models for cancer cachexia in humans. Moreover, the methods used in these studies have not generally been ideal for quantitative evaluation of protein metabolism. Such methods include the use of isolated tissue samples or homogenates incubated in vitro (3, 14), the results of which may not be physiologically meaningful, and even in vivo measurements are often limited to simple measurements of incorporation of radioactively labeled amino acids into protein, without consideration of the precursor pool specific activity, so that absolute rates of protein synthesis cannot be calculated (14, 16). We have therefore made measurements in vivo of rates of protein synthesis in tissues of mice bearing a xenografted tumor which has been shown to cause considerable weight loss (greater than 25%) even though it comprises less than 5% of the animal's body weight and causes only a 15% decrease in food intake (27).

Protein synthesis was measured in vivo by 2 different methods, namely, the constant infusion of [14C]tyrosine (8) and the single injection of a flooding dose of [3H]phenylalanine (9).

Measurements were also made on control mice subjected to varying degrees of food restriction in order to assess the contribution of reduced food intake to the alterations in protein turnover.

A preliminary report of part of this work has been published previously (6).

MATERIALS AND METHODS

Animals

Female CBA/Ca mice obtained from OLAC (Oxford, United Kingdom) were maintained on Spratts Laboratory Rodent Diet at an environmental temperature of 24° throughout the study. At approximately 8 weeks of age, the thymus was surgically removed under ether anesthesia, and 4 weeks later the animals were given injections of cytosine 1-β-D-arabinofuranosyl (200 mg/kg body weight) and then exposed to cobalt radiation (900 rads/mouse). A small piece of tissue (1 cu mm) containing tumor cells derived from a human hypernephroma (XK1; see Ref. 27) was implanted into the right flank of each mouse. Control mice were either immunosuppressed as above but not given implants of the tumor, or were left intact throughout the study.

Approximately 4 weeks after implantation, the tumors began to grow. The mice were then weighed daily, and the measurement of protein synthesis was performed when the tumor-bearing mice had lost at least 20% of their original body weight.

Measurement of Protein Synthesis

Constant Infusion Method. Conscious mice were infused via a lateral tail vein with a solution of i-[U-14C]tyrosine (4 μCi/ml; 0.3 mCi) in aqueous NaCl (9 g/liter) at a constant rate of 0.25 ml/hr. After 4 hr, the mice were decapitated, blood was collected in a heparinized beaker, and samples of liver and muscles (quadriceps and gastrocnemius) from both hind limbs were rapidly removed and frozen in liquid nitrogen. The specific radioactivity of tyrosine in blood plasma and in the free and protein-bound pools of liver and muscle was measured using the specific, enzymatic assay described by Garlick and Marshall (8). Whole-body tyrosine flux, Q, was calculated from:

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where $I$ is the infusion rate, and $Sp$ is the specific radioactivity of $[^{14}C]$tyrosine in the plasma at the end of the infusion. Tissue protein-synthetic rates, $Ks$, were calculated from the simplified equation:

$$Ks = \frac{S_e}{S_i \Delta t}$$

where $S_e$ and $S_i$ are the specific radioactivities of protein-bound and intracellular-free tyrosine, respectively, and $\Delta t$ is the length of the infusion time. We chose 4 hr as the length of the infusion, because preliminary experiments showed that the specific radioactivity of free tyrosine in blood plasma and muscle had reached a plateau after 1 hr and remained constant for the next 3 hr (results not shown).

**Large-Dose Method.** Mice were given injections via a lateral tail vein with a flooding dose (150 mmol and 50 $\mu$Ci in 1 ml/100 g body weight) of L-phenyl[2,3-$^3$H]alanine; 10 min later, they were decapitated and rapidly cooled in iced water, and samples of liver and muscles (quadriceps and gastrocnemius) from both hind limbs were removed and rapidly frozen in liquid nitrogen. The fractional rate of protein synthesis was calculated from the ratio of the specific radioactivities of free and protein-bound phenylalanine measured by the specific, enzymatic assay described by Garlick et al. (9). Tissue protein contents were determined by the method of Lowry et al. (13). RNA content was estimated from absorbance at 260 nm, with appropriate correction for peptide absorbance (20); DNA content was measured by the method of Giles and Myers (11).

Since this method for measuring protein synthesis was originally developed for use in rats, its validity for these studies in mice was first checked. Groups of 8 mice were killed at 2, 5, and 10 min after injection of the flooding dose of $[^{3}H]$phenylalanine. The specific radioactivity of $[^{3}H]$phenylalanine in liver, muscle, and blood plasma was found to fall linearly during the 10 min in all 3 groups of mice (Chart 1). In subsequent experiments, therefore, mice were killed at 10 min after injection of the flooding dose of $[^{3}H]$phenylalanine, and an appropriate correction was made to the measured value for precursor specific radioactivity in calculating the value for protein synthetic rate.

**Dietary Studies**

For these studies, mice were transferred to a semisynthetic diet, (20% casein, 30% maize starch, 30% sucrose, 10% maize oil, 5% cellulose, and appropriate vitamins and minerals), and housed individually in wire-bottomed cages, so that spilled food could be recovered and weighed, after separation from feces by sieving. Eight tumor-bearing and 5 thymectomized control mice were allowed to feed ad libitum until the tumor-bearing mice had lost 20% of their initial body weight, and food intake was measured daily.

**RESULTS**

Thymectomy caused a considerable loss of body weight, presumably due to anorexia during recovery from the operation, but by the time of tumor implantation, the weights of all the immunosuppressed mice had stabilized at 75% of the weight of the intact mice. The food intake of the immunosuppressed mice [0.306 ± 0.011 (S.E.) g/g body weight$^{0.75}$/day] was comparable to that of intact mice fed ad libitum (0.299 ± 0.017 g/g body weight$^{0.75}$/day). After tumor implantation, the weights of the tumor-bearing mice remained stable, and comparable to those of the immunosuppressed control mice, for at least 4 weeks. They then lost 20% of their body weight over a period of 5 days, at which time measurement of protein synthesis was performed (Chart 2). Tumor weight at this time was 706 ± 182 mg (3.3% of body weight). In some mice, the tumor did not grow, and these animals did not lose weight; these mice formed a separate control group (tumor regressing). The body weight of the control mice (intact and immunosuppressed) did not change significantly throughout the study.

Food intake of the tumor-bearing mice did not differ significantly from that of the immunosuppressed control mice for the
4 weeks after tumor implantation. However, during the 5 days in which the tumor-bearing animals lost weight, their food intake was 15% less than that of the control mice (Table 1). This confirms a previous measurement made on these mice using a liquid feed (27). When expressed per unit of metabolic body weight, the food intake of the tumor-bearing mice (0.265 ± 0.016 g/g body weight0.75/day) was only 9% less than that of the control mice at this time (0.291 ± 0.011 g/g body weight0.75/day).

Normal mice subjected to a 15% food restriction lost 7% of their body weight over a 7-day period; mice subjected to a 50% food restriction lost 19% of their body weight over 7 days (Chart 3). Thus, in neither of these groups was the rate of loss of body weight as great as that exhibited by the tumor-bearing mice. Clearly, weight loss in the tumor-bearing mice could not be accounted for by reduced food intake alone.

The results of the constant infusion experiments showed that tyrosine flux was decreased in the tumor-bearing mice as compared with immunosuppressed control mice (Table 2). The largest components of tyrosine flux measured in this way are the removal of tyrosine for protein synthesis and the appearance of tyrosine from protein degradation. Protein synthesis probably accounts for 80 to 85% of tyrosine flux, since oxidation, which is the only other process removing tyrosine from the free pool, has been shown to account for 15 to 20% of the tyrosine flux in humans and rats (12, 25). Similarly, protein degradation probably accounts for 85 to 90% of the tyrosine flux in the postabsorptive state; of the other processes contributing tyrosine to the free pool, dietary input is negligible, since the mice were not fed during the 4-hr infusion, nor for 2 hr previously, and phenylalanine hydroxylation amounts to 10 to 15% of the tyrosine flux in normal humans (4, 12). If these proportions are applied to the control mice in this study, it is clear that the 60% reduction in tyrosine flux observed in the tumor-bearing mice must have involved reductions in the rates of whole-body protein synthesis and whole-body protein degradation.

These changes in whole-body protein turnover were investigated further by measuring rates of protein synthesis in individual tissues.

The constant infusion experiment showed that protein synthesis was decreased by 58% in muscle, although there was no significant change in protein synthesis in liver (Table 2). However, there is some doubt about the validity of values for liver protein synthesis measured by this technique, because of uncertainty as to the true precursor pool for protein synthesis, the possibility of recycling of label, and the loss of the labeled export protein. Therefore, we made further measurements using a flooding dose technique which was designed to overcome these problems in measuring liver protein synthesis.

Protein synthesis in both liver and muscle, measured by the large-dose phenylalanine method, was significantly reduced in the tumor-bearing mice compared with all the control groups (Tables 3 and 4). The very severe reduction in protein synthesis in muscle (to 30% of the thymectomized control value) was partly due to a 20% loss of RNA; however, when the protein synthetic activity of RNA was calculated (by dividing the fractional synthetic rate by the RNA:protein ratio), this value was also markedly reduced. Reductions in both RNA content and RNA activity in muscle have been reported as part of the adaptive response to various regimes of nutritional deprivation (10). The reduction in protein synthesis in the liver was less severe than that in muscle (to 60% of the thymectomized control value), and was accounted for entirely by a reduction in RNA activity, with no change in RNA content. A similar response has been observed in rats fed a protein-free diet (17) or starved for 2 days (10).

Normal mice subjected to dietary restrictions showed reductions in the rates of protein synthesis in both liver and muscle, results qualitatively similar to those found in tumor-bearing mice (Table 5). However, the extent of the reduction in protein synthesis which could be achieved by food restriction in normal mice by 15 or 50% was much less than that observed in tumor-bearing mice. Only in mice which had been fasted for 48 hr was the fall in protein synthesis in liver and muscle as severe as that observed in tumor-bearing mice. Of course, dietary restriction of normal mice is not a perfect model for the anorexia induced by cancer, since the food-restricted mice would probably have consumed all their food more rapidly than the tumor-bearing mice. Moreover, our measurements were all made at approximately 11 a.m., after withdrawing the animals' food at 9 a.m., so that the results refer only to the postabsorptive state.
These tissues constitute the largest components of whole-body and the ratio of DNA to protein increased, the ratio of RNA to concentration of protein (per unit wet weight) remained unchanged while the total amounts of both RNA and DNA were maintained, while cachexia. Most of the major organs of the body, including liver to be a useful experimental model for the study of cancer more severe in muscle than in liver. Nevertheless, these results indicate that anorexia alone is unlikely to account entirely for the markedly depressed rates of protein synthesis in liver and muscle exhibited by the tumor-bearing mice.

**DISCUSSION**

Previous work (27) has shown the XK1 tumor-bearing mouse to be a useful experimental model for the study of cancer cachexia. Most of the major organs of the body, including liver and 2 representative skeletal muscles, were reported to lose weight in proportion to the loss of host body weight. In the liver, the total amounts of both RNA and DNA were maintained, while protein was lost; these results are confirmed by our present findings. In muscle, the situation is different. Although the concentration of protein (per unit wet weight) remained unchanged and the ratio of DNA to protein increased, the ratio of RNA to protein decreased. This considerable loss of RNA was partly responsible for the fall in the rate of protein synthesis, which was more severe in muscle than in liver.

Protein synthesis was measured in liver and muscle, since these tissues constitute the largest components of whole-body protein synthesis in small animals (18). Protein metabolism in quadriceps and gastrocnemius muscles is believed to be representative of protein metabolism in the musculature as a whole (31). We found reduced rates of protein synthesis in both liver and muscle in XK1 tumor-bearing mice as well as a decrease in whole-body amino acid flux. These results indicate that protein metabolism in mice during tumor-induced cachexia changed in the same way as that in rats undergoing varying degrees of dietary restriction (10, 18). However, the reductions in protein synthesis in liver and muscle and the rate of loss of body weight were greater in the cancer-bearing mice than in control mice whose food intake was reduced by more than the spontaneous reduction in food intake observed in the XK1 tumor-bearing mice. The reduction in protein synthesis in normal animals in response to dietary restriction is accompanied by an adaptive fall in the rate of protein breakdown, which tends to minimize the rate of loss of body tissues while supplying amino acid precursors for gluconeogenesis (10). The greater reduction in tissue protein synthesis rates caused by the presence of the tumor, which was associated with a rapid loss of weight, may have been due to the specific need of the tumor for glucose as an obligatory fuel, stimulating demand for gluconeogenic precursors (29). Alternatively, it has been suggested that the presence of the tumor directly causes an increase in the rate of fuel oxidation in brown adipose tissue (1), and that the wasting of other tissues is then caused by the demand of brown adipose tissues for metabolic substrates. The possibility remains, however, that the depression of muscle and liver protein synthesis is caused by a hormone-like substance secreted by the tumor, and that the increase in brown adipose tissue metabolism is simply a consequence of increased substrate availability from the wasting of other tissues. We reported previously that the concentration of free 3-methylhistidine was increased by 80% in the muscles of the tumor-bearing mice, and interpreted this as indicating an increase in the rate of muscle protein breakdown (6). However, we now know that such increases in free 3-methylhistidine concentration

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**Table 3**

| Tumor-bearing (n = 11) | 3.1 ± 1.6 | 160 ± 3 | 7.1 ± 0.2 | 4.1 ± 0.2 | 4.2 ± 2.0 |
| Thymectomized control (n = 8) | 10.2 ± 2.5 | 155 ± 5 | 8.8 ± 0.3 | 2.8 ± 0.1 | 12.2 ± 3.4 |
| Intact control (n = 9) | 9.5 ± 3.8 | 140 ± 5 | 9.8 ± 0.4 | 3.1 ± 0.1 | 10.8 ± 4.5 |
| Tumor-regressing (n = 4) | 10.4 ± 3.3 | 147 ± 3 | 7.9 ± 0.3 | 2.6 ± 0.1 | 14.3 ± 6.8 |

| Tumor-bearing (n = 10) | 73 ± 12 | 163 ± 3 | 84.0 ± 1.8 | 28.3 ± 1.2 | 8.6 ± 1.2 |
| Thymectomized control (n = 7) | 130 ± 12 | 154 ± 6 | 75.8 ± 2.1 | 18.3 ± 0.4 | 17.1 ± 1.5 |
| Intact control (n = 6) | 115 ± 13 | 146 ± 6 | 72.2 ± 1.4 | 19.7 ± 0.3 | 16.0 ± 1.7 |
| Tumor-regressing (n = 4) | 117 ± 8 | 124 ± 4 | 80.9 ± 2.0 | 16.8 ± 1.0 | 14.8 ± 1.2 |

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**Table 4**

| Tumor-bearing (n = 10) | 73 ± 12 | 163 ± 3 | 84.0 ± 1.8 | 28.3 ± 1.2 | 8.6 ± 1.2 |
| Thymectomized control (n = 7) | 130 ± 12 | 154 ± 6 | 75.8 ± 2.1 | 18.3 ± 0.4 | 17.1 ± 1.5 |
| Intact control (n = 6) | 115 ± 13 | 146 ± 6 | 72.2 ± 1.4 | 19.7 ± 0.3 | 16.0 ± 1.7 |
| Tumor-regressing (n = 4) | 117 ± 8 | 124 ± 4 | 80.9 ± 2.0 | 16.8 ± 1.0 | 14.8 ± 1.2 |

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**Table 5**

<table>
<thead>
<tr>
<th>Protein synthesis measured by the large-dose [3H]phenylalanine method in liver and muscle of normal mice undergoing dietary restriction</th>
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<tbody>
<tr>
<td>Liver (%/day)</td>
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<tr>
<td>Control (n = 5)</td>
</tr>
<tr>
<td>15% restricted (n = 5)</td>
</tr>
<tr>
<td>50% restricted (n = 5)</td>
</tr>
<tr>
<td>Control (n = 4)</td>
</tr>
<tr>
<td>48-hr fasted (n = 4)</td>
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</tbody>
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*Mean ± S.E.

* Significance different from thymectomized control value; p < 0.01.
in muscle may be secondary to increases in blood plasma following increases in protein breakdown in other tissues, including gut, particularly under circumstances of reduced food intake (24). The tumor-bearing mice lost 6.6% of their body weight during the 24 hr preceding the measurement of protein synthesis. Assuming that the total weight of quadriceps and gastrocnemius muscles fell at the same rate as did host body weights, as indicated by Strain et al. (27), and that the concentration of protein in these muscles remained constant (Table 3), this implies that the net rate of loss of muscle protein at this time was approximately 6.6%/day. Since the rate of protein synthesis in these animals' muscles was 3.1%/day (Table 3), the rate of muscle protein degradation would have been approximately 9.7%/day. The rate of muscle protein degradation in the thymectomized control mice would have been approximately equal to the rate of muscle protein synthesis, 10.2%/day, since these animals were neither gaining nor losing weight. It seems, therefore, that the tumor-bearing animals lose muscle protein by a reduction in the rate of protein synthesis below the nearly constant rate of protein degradation, as has been shown for a variety of muscle wasting conditions caused by disease or malnutrition in experimental animals and humans (22).

The above calculation does not yield exact values for the rate of protein degradation in muscle, because we did not determine the total protein content of quadriceps and gastrocnemius muscles of matched groups of mice on successive days of the experiment. The most likely source of error is that the mice lost fat more rapidly than protein, as was suggested by Strain et al. (27). It is unlikely that the 20% loss of body weight was due entirely to lipid depletion, since normal adult mice only contain approximately 15% body fat (19), and Strain et al. (27) reported significant losses of weight from several organs which contain very little fat. Nevertheless, if there was significant lipid depletion, so that muscle protein was lost more slowly than is assumed above, then the calculated rate of muscle protein degradation was further decreased.

The only other reported measurements of protein synthesis in tumor-bearing animals made by reliable methods in vivo showed a decrease in protein synthesis in muscle but an increase in protein synthesis in liver of mice which had been given injections of Ehrlich ascites (21). The increased rate of protein synthesis in the livers of these animals resulted in increased liver weight. Other workers have reported increased (26) or unchanged (16) liver weight in tumor-bearing animals, although all these studies have used animals with tumor burdens considerably greater than that of the XK1 tumor. There have been reports of both increased (28) and decreased (26) liver weights from postmortem analyses of cachectic cancer patients. It may be that the effect of cancer in other tissues on protein metabolism in the liver varies with the progression of the disease and the size of the tumor burden.

Liver protein synthesis was reduced by 44% when measured by the large-dose phenylalanine method, whereas the reduction measured by the constant infusion method (29%) was not statistically significant. This indicates a greater reduction in the rate of synthesis of export protein than in the rate of synthesis of fixed liver protein, since the incorporation of label after 4 hr of constant infusion will tend to reflect to a greater extent the synthesis of fixed liver proteins, while the incorporation during the 10 min of the large-dose method will reflect the synthesis of both types of protein.

We have shown that the loss of body protein from mice bearing the XK1 tumor is mediated by a reduction in the rate of protein synthesis in liver and muscle tissues, and this is likely to be the case in cachectic cancer patients as well. We have preliminary evidence that muscle protein synthesis, measured by \[^{15}\text{C}\]leucine infusion (23), is indeed reduced in cachectic cancer patients. This would fit with a large body of evidence that many muscle-wasting conditions are associated with reduced protein synthesis in muscle (22). This implies that nutritional and pharmacological interventions designed to stimulate protein synthesis may be an appropriate part of the management of cachectic cancer patients, and we have recently reported that enteral feeding stimulates protein synthesis in muscle, liver, and gut, but not the tumor, of rats with colorectal cancer (7).

ACKNOWLEDGMENTS

We are grateful to Professor R. H. T. Edwards and Professor A. M. Neville for enthusiastic support and advice.

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JULY 1984 2783
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