Perturbations of triglycerides but not of cholesterol metabolism are prevented by anti-tumour necrosis factor treatment in rats bearing an ascites hepatoma (Yoshida AH-130)

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Summary Rats transplanted with the ascites hepatoma Yoshida AH-130 developed a severely progressive cachexia, characterised by marked alterations in protein and lipid metabolism. In particular, high levels of serum triglycerides and free fatty acids were associated with altered levels and distribution of plasma cholesterol, with increased total and very low-density lipoprotein–low-density lipoprotein (VLDL–LDL) cholesterol and reduced high-density lipoprotein (HDL) cholesterol. The tumour cells showed high rates of cholesterol synthesis and elevated plasma lipoprotein lipase activity, which, in turn, was reduced in the host liver. To determine whether these perturbations could be related to the elevation of tumour necrosis factor α (TNF-α) previously shown in the AH-130 bearing (Tessitore L, Costelli P, Baccino FM 1993, Br J Cancer, 67, 15–23), either anti-TNF polyclonal antibodies or non-immune IgGs were injected daily after tumour transplantation. The anti-TNF treatment neither affected tumour growth nor prevented the serum cholesterol changes, while attenuating the hypertriglyceridaemia and the elevated serum free fatty acid levels. These data indicate that TNF does not appear to be directly involved in the altered cholesterol metabolism in AH-130 hosts, thus supporting the view that cholesterol metabolism and lipid metabolism are regulated differently during tumour growth.

Keywords: tumour growth; tumour necrosis factor; anti-tumour necrosis factor; lipid metabolism; cholesterol metabolism

In the past few years a large body of evidence has been accumulated indicating a possible central role of cholesterol in the pathobiology of cancer. Alterations in the synthesis, uptake and intracellular content of cholesterol have been observed in a variety of experimental tumour models as well as in different types of human neoplasms (Anderson et al., 1981; Coleman and Lavietes, 1981; Yachnin et al., 1983; Dessi et al., 1992a,b, 1994). Cholesterol perturbations also include change in lipoprotein profiles in plasma compartment. In particular, a definite decrease in high-density lipoprotein (HDL) levels is a consistent finding in both experimental rat tumours and human neoplasms (Dessi et al., 1991, 1992a,b, 1994) despite the differences reported to exist between lipoprotein metabolism in rodents and humans (Dietschy et al., 1993). In contrast, changes in other serum lipid parameters, such as total cholesterol and triglyceride levels, appear to vary and to be species specific and dependent on the histological type or tumour grade (Dessi et al., 1991, 1992a,b, 1994). Therefore, it is possible that the mechanistic basis for the altered HDL levels is different from that responsible for the observed changes in other serum lipid parameters. Admittedly, HDLs play an important role in the transport of excess cholesterol from extrahepatic tissues to the liver for reutilisation or excretion into bile (reverse cholesterol transport). It is thus conceivable that the observed low levels of HDL-cholesterol may be related, at least in part, to a decreased cholesterol efflux to HDL as a consequence of increased utilisation and storage in actively proliferating tissues, such as neoplasms. However, since precursor particles of HDL are thought to derive from lipolysis of triglyceride rich lipoproteins (Eisenberg, 1984) and since a significant positive correlation between plasma HDL cholesterol and lipoprotein lipase (LPL) activity in adipose tissue has also been reported (Eisenberg, 1984), the possibility that low HDL cholesterol concentrations observed during tumour growth may be secondary to the decreased triglyceride clearance from plasma, as a result of LPL inhibition, must also be considered.

Tumour necrosis factor (TNF), a pleiotropic cytokine primarily produced by activated macrophages in response to invasive stimuli (Beutler and Cerami, 1988), has been frequently reported as being responsible for changes in lipid metabolism which occur in association with infections and tumours in a wide variety of species, including humans and rats (Feingold et al., 1987, 1992; Harada et al., 1990). TNF might affect plasma cholesterol, triglyceride and lipoprotein levels by both inhibition of adipose LPL activity and/or stimulation of hepatic lipogenesis (Feingold et al., 1992, 1993).

Lipid metabolism in rats bearing ascites hepatoma Yoshida AH-130 has been previously investigated in our laboratories. This tumour causes in the host a rapid loss of body weight, associated with marked perturbations of both protein (Tessitore et al., 1987, 1993) and lipid metabolism (Dessi et al., 1992a). Increased synthesis and progressive accumulation of cholesterol were observed in AH-130 cells. During tumour growth, rats developed changes in serum lipid concentrations that included elevation of total cholesterol and triglycerides as well as a sharp reduction of HDL-cholesterol. Concurrently, the plasma levels of TNF were elevated, while the activity of LPL in the white adipose tissue was decreased (Tessitore et al., 1993; Carbó et al., 1994).

Based on these results, the aim of the present study was to investigate whether and to what extent TNF may mediate changes in lipid metabolism in AH-130-bearing rats. The results obtained using passive immunisation against TNF seem to indicate that the TNF produced during tumour growth altered triglyceride and free fatty acid metabolism, yet was not involved in cholesterol metabolism perturbations.
Materials and methods

Animals and tumours

The study was performed on male Wistar rats (Charles River, Como, Italy), weighing about 150 g. They were maintained on a regular dark-light cycle (light 8 a.m.–8 p.m.) and free access to food (Piccioni, Brescia, Italy) and water. The rats were divided into four groups, namely controls, tumour hosts and tumour hosts treated with either non-immune IgGs or anti-TNF antibody. The Yoshida ascites hepatoma cells (approximately 10⁶ cells per rat) were inoculated intraperitoneally (for details see Tesitore et al., 1987). The daily food intake of all groups of animals was measured.

Treatments

Two groups of tumour hosts received daily a subcutaneous injection of 25 mg kg⁻¹ body weight of a polyclonal goat anti-murine TNF IgG preparation (anti-TNF) or of a non-immune goat IgG preparation (IgGs), as previously described (Bagby et al., 1991; Costelli et al., 1993). The treatment started the day after transplantation and lasted 3 days. Animals were killed on day 4 under light ether anaesthesia.

DNA synthesis

To measure DNA synthesis Yoshida AH-130 cells obtained from the three groups of tumour hosts were incubated in the presence of [³H]thymidine as previously reported (Dessi et al., 1992a). Briefly, 1 × 10⁸ tumour cells were placed in glass tubes containing Krebs' bicarbonate buffer and 10 μCi of [³H]thymidine (25 Ci mmol⁻¹, from New England Nuclear, Boston, MA, USA) in an atmosphere of 95% oxygen–5% carbon dioxide, and incubated at 37°C for 2 h. The cells were then recovered on glass filters using an automatic harvester (Flow, Irvine, UK) and radioactivity counted in a liquid scintillation spectrometer (Beckman, USA), using Ultima Gold as scintillation fluid (Packard, Moriden, CT, USA).

Cholesterol and triglyceride synthesis

The rate of cholesterol and triglyceride synthesis was determined by measuring the in vitro incorporation of [¹⁴C]acetate in both liver and AH-130 tumour cells. Livers were cut into thin slices (1 mm thick) and tumour cells processed as described above. For the assay 500 mg of tissue slices or 2 × 10⁷ tumour cells were placed in glass tubes containing Krebs' bicarbonate buffer and incubated with 10 μCi of [¹⁴C]acetate (New England, Nuclear, Boston, MA, USA, sp. ac. 45–60 mCi mmol⁻¹) for 2 h at 37°C in an atmosphere of 95% oxygen–5% carbon dioxide. After incubation, the tissue slices and the cells were washed twice, homogenated, and lipids extracted with chloroform–methanol (2:1) according to Folch et al. (1957). After evaporation of the solvent, the lipids were dissolved in chloroform and neutral lipids were separated by thin-layer chromatography (DC-Alufolien Kieselgel 60, Merck, Darmstadt, Germany), using the solvent system n-heptane–isopropanol–formic acid (60:40:2, v/v/v). The bands corresponding to free and esterified cholesterol and triglycerides were then visualised using iodine vapour and scraped into counting vials to detect the incorporation of [¹⁴C]acetate (Bowman and Wolf, 1962; Van Handel and Zilversmit, 1968).

Separation of lipoproteins by high-performance liquid chromatography (HPLC)

Serum lipoproteins were separated by HPLC according to Okazaky et al. (1980). The analyses were carried out on a Series 4 Perkin-Elmer liquid chromatograph equipped with LC-85B Perkin-Elmer variable wavelength UV detector and a II Series 3396 Hewlett-Packard integrator. An aliquot of 10 μl of serum was injected and sodium chloride (pH 6.96; 0.15 M) was used as eluant. The columns were gel permeation chromatography (GPC) columns filled with microspheres of hydrophilic polymers with an aqueous support based on a chemically modified silica (TSK GEL, Toyo Soda, Tokyo). Each column was 600 × 7.5 mm i.d. For better resolution of lipoprotein subfraction peaks, a combination of GPC columns (G5000PW + G3000SW × 2) was used. To increase the lifespan of the columns a guard column was inserted. The columns were balanced with very low density lipoprotein (VLDL), low-density lipoprotein (LDL), HDL₁ and HDL₃ standard prepared by ultracentrifugation according to the method of Havel et al. (1955). Proteins in VLDL, LDL, HDL₁ and HDL₃ subfractions were monitored by absorbance at 280 nm.

Analytical procedures

To determine free and esterified cholesterol as well as triglyceride contents, total lipids were extracted as described above. The two cholesterol moieties were measured as directed by Bowman and Wolf (1962), using cholesterol and cholesterol palmitate (Sigma, St Louis, MO, USA) as standards, while triglyceride content was evaluated by the method of Van Handel and Zilversmit (1968), with triolein as the working standard.

DNA content was measured by the method of Boer (1975) and protein that of Lowry et al. (1951), using herring sperm DNA and bovine serum albumin as working standards respectively.

Cholesterol, triglyceride, free fatty acid and phospholipid concentrations in plasma and ascitic fluid were estimated using commercially available kits (Bohrenger, Mannheim, Germany). VLDL and LDL were isolated by precipitation with a mixture of phosphotungstic acid and magnesium ions. After standing for 10 min at room temperature the mixtures were centrifuged at 10 000 g for 10 min. The supernatant containing the HDL fraction was removed and the levels of cholesterol, triglyceride and phospholipid were determined. The precipitate containing the VLDL–LDL fraction was dissolved in 0.15 M sodium chloride and the cholesterol, triglycerides and phospholipids were assayed as above.

Statistical analysis

Significance of the differences was calculated by the Student's t-test.

Results

The ascites hepatoma Yoshida AH-130 caused a loss of body weight in tumour hosts, in association with the presence of detectable levels in circulating TNF. A decrease in daily food intake was also observed in tumour-bearing rats (Table I). The results are in agreement with our previous studies which have demonstrated that ascites hepatoma AH-130 causes in the rat host a rapid and progressive loss of body weight, a progressive decline of food intake, skeletal muscle waste and lipid depletion (Costelli et al., 1993; Carbó et al., 1994). The anti-TNF treatment effectively neutralised circulating TNF, which either the anti-TNF antibodies nor non-immune IgGs modified tumour growth, body weight loss and food intake decline (Table I).

The hepatic synthesis of total cholesterol and triglycerides is shown in Table II. The synthesis of total cholesterol was reduced during tumour growth, while no changes were observed for triglycerides, in keeping with previous observations (Dessi et al., 1992a). The anti-TNF treatment did not modify either parameter.

High synthesis rates for cholesterol, both free and esterified, and for triglycerides have been observed in the AH-130 cells (see Dessi et al., 1992a) and were not modified by the anti-TNF treatment (Table III). Consistently, total and free cholesterol and triglyceride levels in tumour cells were not affected by the treatment (Table IV). Moreover,
Table I  Body weight, food intake, tumour growth, and plasma TNF in AH-130 tumour bearing rats

| Animals and treatment | Body weight (g) | Food intake (g) | Tumour cells (× 10⁴) | [³H]Thymidine incorporation (d.p.m. μg DNA) | TNF (pg ml⁻¹) |
|-----------------------|-----------------|-----------------|----------------------|------------------------------------------|----------------|
| Controls              | 147 ± 5a        | 22 ± 2b         | -                    | -                                        | ND             |
| AH-130 hosts          |                 |                 |                      |                                          |                |
| None                  | 125 ± 9a        | 16 ± 1b         | 1458 ± 208a          | 6198 ± 521a                              | 88 ± 4a        |
| IgGs                  | 117 ± 2a        | 15 ± 1b         | 1678 ± 261b          | 5946 ± 499b                              | 80 ± 7b        |
| Anti-TNF              | 116 ± 3a        | 15 ± 1b         | 1705 ± 401b          | 6578 ± 320b                              | ND             |

Values are mean ± s.e.m. Body weight in AH-130 hosts is exclusive of tumour. ND, not detectable.

Means with different letters are significantly different (P<0.01).

Table II  Cholesterol and triglyceride synthesis in liver of AH-130 tumour-bearing rats

| Animals and treatment | [¹⁴C]Acetate incorporated into cholesterol (c.p.m. 100 mg⁻¹ liver) | [¹⁴C]Acetate incorporated into triglycerides (c.p.m. 100 mg⁻¹ liver) |
|-----------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| Controls              | 1857 ± 85a                                                    | 1445 ± 244a                                                  |
| AH-130 hosts          |                                                             |                                                              |
| None                  | 748 ± 103b                                                    | 1047 ± 64b                                                   |
| IgGs                  | 1191 ± 164b                                                   | 1413 ± 267b                                                  |
| Anti-TNF              | 1089 ± 137b                                                   | 1414 ± 437b                                                  |

Values are mean ± s.e.m. Means with different letters are significantly different (P<0.01).

Discussion

Previous studies by our laboratories (Dessi et al., 1992a) as well as the present one have revealed that rats bearing ascites hepatoma AH-130 are characterised by a specific pattern of lipid metabolism. Cholesterol synthesis and content were high in AH-130 cells, triglycerides and total cholesterol increased in the host plasma, while esterified cholesterol was decreased to about 50% compared with control values. Analysis of plasma lipoproteins revealed an elevation of VLDL and LDL in host rats compared with control animals, with more than a 3-fold increase in both lipid and protein content. In contrast HDL, in particular the HDL₂ subfraction, was reduced. These alterations were associated with marked perturbations in the hormonal homeostasis and presence of detectable levels of circulating TNF (Tessitore et al., 1993).

Changes in lipid metabolism are a common feature during neoplastic growth, both in humans and in different experimental model systems (Clark and Cranin, 1986; Dessi et al., 1986, 1989, 1992a,b, 1994). However, the mechanisms underlying these changes are still unclear and complicated by the fact that the metabolic alterations are species specific and dependent on the histological type or the degree of malignancy.

In this report, our results on serum cholesterol and lipoprotein levels in tumour-bearing rats were similar to those previously observed by other investigators in rats and mice (Kannan and Baker, 1977; Clark and Cranin, 1986), but in contrast with those reported in humans (Rosner and Wallgren, 1984; Vitols et al., 1985; Bani et al., 1986; Reverter et al., 1988; Dessi et al., 1991, 1992b, 1994). In particular, normal or decreased serum cholesterol levels were observed in cancer patients, while triglyceridaemia appears variable, dependent on the type of tumours and age of appearance.

Nevertheless, a decrease in circulating HDL levels is found in virtually all neoplastic and inflammatory diseases studied in both rodents and humans (Dessi et al., 1986, 1989, 1991, 1992a,b, 1994; Feingold et al., 1993) suggesting that the mechanisms underlying changes in total cholesterol and triglyceridaemia are presumably different from those responsible for lowering HDL levels.

Based on our data, two mechanisms can be considered to explain the increase in circulating lipids in AH-130 hosts. First, an increased mobilisation of lipids from fat depots, as evidenced by the loss of body weight and the increase in serum free fatty acids. Second, a decrease in the clearance of VLDL as evidenced by the decrease in LPL activity in adipose tissue previously observed in this type of tumour (Carbó et al., 1994). Under our experimental conditions, it is unlikely that diet and endogenous biosynthesis can be responsible for the observed hyperlipidaemia in that both hepatic lipid synthesis and food intake, the two main sources of plasma lipids in the body, were normal or decreased in tumour-bearing rats. TNF, a pleiotropic cytokine, is primarily produced by activated macrophages in response to invasive stimuli (Beutler and Cerami, 1988). As for lipid homeostasis, TNF has been shown to activate peripheral lipolysis and hepatic...
Table III  Cholesterol and triglyceride synthesis in AH-130 cells

| Treatment | n  | Total cholesterol | Free Cholesterol | Esterified Cholesterol | Total triglycerides | Free triglycerides | Esterified triglycerides |
|-----------|----|-------------------|------------------|------------------------|--------------------|-------------------|-------------------------|
| None      | 9  | 56.2 ± 8.44      | 56.2 ± 8.44      | 254 ± 13.34           | 184 ± 18.2          | 184 ± 18.2         | 184 ± 18.2              |
| IgGs      | 5  | 43.7 ± 3.44      | 43.7 ± 3.44      | 209 ± 25.25           | 182 ± 14.6          | 182 ± 14.6         | 182 ± 14.6              |
| Anti-TNF  | 5  | 59.5 ± 2.2      | 59.5 ± 2.2       | 222 ± 18.3            | 199 ± 28.2          | 199 ± 28.2         | 199 ± 28.2              |

Values are mean ± s.e.m. *Means with the same letter are not significantly different.

Table IV  Cholesterol, lipid and protein content in AH-130 cells

| Treatment | n | Cholesterol (mg/dl) | Triglycerides (mg/dl) | Phospholipids (mg/dl) | Free fatty acid (mequiv. I.) | Proteins (mg/ml) |
|-----------|---|---------------------|-----------------------|----------------------|-----------------------------|-----------------|
| None      | 9 | 55.8 ± 2.74       | 3.7 ± 0.74            | 28.8 ± 5.12          | 56.4 ± 3.95                 |                 |
| IgGs      | 5 | 39.9 ± 2.76       | 15.5 ± 1.17           | 17.6 ± 1.7           | 40.0 ± 3.8                  |                 |
| Anti-TNF  | 5 | 38.8 ± 6.00       | 19.7 ± 0.76           | 14.6 ± 2.8           | 35.9 ± 4.0                  |                 |

Values are mean ± s.e.m. *Means with different letters are significantly different (tP < 0.05 vs controls; tP < 0.05 vs untreated AH-130 hosts).

Table V  Cholesterol, lipid and protein content in serum of AH-130 tumour-bearing rats

| Animals and treatment | n | Cholesterol (mg/dl) | Triglycerides (mg/dl) | Phospholipids (mg/dl) | Free fatty acid (mequiv. I.) | Proteins (mg/ml) |
|-----------------------|---|---------------------|-----------------------|----------------------|-----------------------------|-----------------|
| Controls              | 4 | 55.8 ± 2.74       | 3.7 ± 0.74            | 28.8 ± 5.12          | 56.4 ± 3.95                 |                 |
| AH-130 hosts          |   |                    |                       |                      |                             |                 |
| None                  | 9 | 39.9 ± 2.76       | 15.5 ± 1.17           | 17.6 ± 1.7           | 40.0 ± 3.8                  |                 |
| IgGs                  | 5 | 38.8 ± 6.00       | 19.7 ± 0.76           | 14.6 ± 2.8           | 35.9 ± 4.0                  |                 |
| Anti-TNF              | 5 | 42.8 ± 4.00       | 14.4 ± 1.73           | 15.8 ± 3.0           | 53.8 ± 5.0                  |                 |

Values are mean ± s.e.m. *Means with different letters are significantly different (tP < 0.01, tP < 0.05 vs controls; tP < 0.05 vs untreated AH-130 hosts).

Table VI  Cholesterol, lipid and protein composition of HDL lipoproteins in AH-130 tumour-bearing rats

| Animals and treatment | n | Cholesterol (mg/dl) | Triglycerides (mg/dl) | Phospholipids (mg/dl) | Proteins (mg/ml) |
|-----------------------|---|---------------------|-----------------------|----------------------|-----------------|
| Controls              | 4 | 21.6 ± 1.32       | 85.2 ± 8.34           | 10.4 ± 0.9           | 5.4 ± 0.3       |
| AH-130 hosts          |   |                    |                       |                      |                 |
| None                  | 9 | 52.7 ± 3.59       | 247.0 ± 33.86         | 41.1 ± 3.77          | 9.5 ± 0.5       |
| IgGs                  | 5 | 37.5 ± 2.39       | 354.0 ± 14.24         | 52.5 ± 2.57          | 11.9 ± 2.2      |
| Anti-TNF              | 5 | 41.0 ± 2.22       | 177.0 ± 23.32         | 50.1 ± 4.40          | 10.5 ± 0.9      |

Values are mean ± s.e.m. *Means with different letters are significantly different (tP < 0.01, tP < 0.05 vs controls; tP < 0.05 vs untreated AH-130 hosts).

lipogenesis, resulting in increased concentrations of circulating lipids (Feingold and Grunfeld, 1987; Starnes et al., 1988; Evans et al., 1989; Grunfeld et al., 1989). In the adipose tissue this cytokine inhibits the synthesis of LPL (Kawakami et al., 1982) as well as the synthesis of acetyl-CoA carboxylase (Pape and Kim, 1988). Fatty acid synthetase (Pekala et al., 1983), fatty acid binding protein and glycerol phosphate dehydrogenase (Torti et al., 1985), all of which are involved in lipid synthesis. TNF also stimulates triglyceride degradation in adipocytes by activating the hormone-sensitive lipase (Pekala et al., 1984).

In vivo administration of this cytokine results in decreased LPL activity in the adipose tissue (Semb et al., 1987; Evans and Williamson, 1988). In a recent study by Ettinger et al. (1990), total plasma cholesterol was reported to be decreased in monkeys treated with TNF (or LPS), due to a reduction in both LDL and HDL fractions and in association with low lecithin-cholesterol acyltransferase activity; moreover, plasma triglycerides were increased, as commonly observed after TNF administration. By contrast, other authors have observed increased serum cholesterol levels after TNF administration (Feingold and Grunfeld, 1987).

These observations support the conclusion that TNF may be involved in the alterations of lipid metabolism that affect
the AH-130 tumour bearers (Carbò et al., 1994). However, treatment with anti-TNF only partially corrected tumour-induced perturbations of lipid metabolism. In particular, triglyceridaemia and free fatty acid levels were reduced, while anti-TNF treatment did not affect total cholesterol levels and HDL levels.

Cell proliferation, either normal or neoplastic, is commonly associated with altered cholesterol metabolism, and in particular with decreased plasma HDL-cholesterol levels (Dessi et al., 1986, 1989, 1992b). It has been proposed that this reduction results from a decrease in the release of cholesterol from proliferating cells to HDL (Daniels et al., 1987). Exogenous cholesterol and cell growth rate modulate the activity of specific HDL receptors; the binding with HDL promotes selective removal of excess cholesterol from the intracellular pool (Oram et al., 1987). It has been shown that both HDL-mediated efflux and HDL receptor activity are down-regulated in actively proliferating cells (Bierman et al., 1989), likely resulting in the reduction of HDL-cholesterol plasma levels.

In the present paper we demonstrate that the anti-TNF treatment is unable to either modify the growth rate of the AH-130 hepatoma or to prevent the decrease in plasma HDL-cholesterol, further supporting the existence of a close relationship between these two parameters.

On the whole, these observations suggest that the hypertriglyceridaemia and the increase of VLDL and LDL, and the increase of HDL metabolism in rats bearing the AH-130 tumour are regulated, at least in part, by different mechanisms. Decreased LPL activity mediated by TNF could well account for the former. By contrast, TNF does not appear to be directly involved in the perturbations of HDL metabolism, which seem to be strictly related to tumour proliferation rates.

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