PHARMACOLOGY AND CELL METABOLISM

Interleukin-15 and Other Myokines in Chronic Alcoholics

Emilio González-Reimers1,*, Camino M. Fernández-Rodríguez1, Francisco Santolaria-Fernández1, María José de la Vega-Prieto2, Candelaria Martín-González1, M. Ángeles Gómez-Rodríguez3, M.R. Alemán-Valls1 and Melchor Rodríguez-Gaspar1

1Servicio de Medicina Interna. Hospital Universitario de Canarias. La Laguna, Tenerife, Spain. 2Servicio de Laboratorio. Hospital Universitario de Canarias. La Laguna, Tenerife, Canary Islands, Spain and 3Servicio de Medicina Nuclear. Hospital Universitario de Canarias. La Laguna, Tenerife, Canary Islands, Spain

*Corresponding author. Tel.: +34-922-678000; E-mail: egonrey@ull.es

(Received 5 March 2011; in revised form 22 April 2011; accepted 3 May 2011)

Abstract — Aims: Interleukin (IL)-15 is highly expressed in skeletal muscle, where it exerts anabolic effects, increasing protein content in muscle fibres and promoting muscle growth. Alcoholics frequently suffer myopathy. Therefore, we analyse the behaviour of IL-15 (and other myokines, such as IL-6, IL-8 and tumour necrosis factor α (TNF-α)) in alcoholics. Methods: These myokines and also malondialdehyde (MDA)—a lipid peroxidation product—were determined by radioimmunoanalytic techniques in blood samples of 35 chronic alcoholics and 13 age- and sex-matched controls, and compared with body composition, nutritional status, liver function, amount of ethanol and routine biochemical variables. Results: IL-15, IL-6, TNF-α, IL-8 and MDA were all higher in alcoholics than in controls; MDA and IL-6 were clearly related with liver function impairment and short-term prognosis, whereas IL-15 was higher among those who died and was related to serum bilirubin. No relation was found between IL-15 and lean mass. Conclusion: IL-15 levels were higher in alcoholics than in controls, especially among those who died within 18 months after admission. They are not related with muscle mass, intensity of alcoholism or nutritional status, but only with serum bilirubin. IL-6 showed inverse correlations with liver function, intensity of alcoholism, nutritional status, left arm muscle mass and short-term mortality.

INTRODUCTION

Interleukin (IL)-15 is a relatively newly described cytokine, highly expressed in skeletal muscle. IL-15 mRNA levels are highly up-regulated in response to strength training, and it seems to exert anabolic effects, increasing protein content in muscle fibres (Furmanczyk and Quinn, 2003) and promoting myogenic differentiation and muscle growth. It would exert an opposite effect to that of tumour necrosis factor α (TNF-α), since it is able to antagonize muscle protein breakdown in a cancer cachexia model (Carbó et al., 2000). Since its discovery in 1994 (Grabstein et al., 1994), it has become clear that it is secreted by many tissues, such as muscle, kidney, heart, lung, dendritic cells, monocytes and macrophages and also enterocytes (Van Heel, 2006), but muscle is the major site of IL-15 mRNA transcription and probably secretion (Grabstein et al., 1994). In addition to its actions on muscle, it also exerts many other functions on memory T cells, γδ T cells, natural killer (NK) T cells, eosinophils, neutrophils, monocytes and macrophages (Quinn, 2008), and shares stimulatory actions on T-cells with IL-2, partly due to the similarity of their respective receptors. Indeed, the heterotrimeric IL-15 receptor is composed of beta and gamma chains identical to those of the IL-2 receptor, together with a specific alpha chain. It is therefore not surprising that it acts as a potent activator of T and B lymphocytes. It is also involved in the maintenance of T cell memory and in the activation of other immune cells, such as neutrophils and NK cells (Fehniger and Caligiuri, 2001). It may be also related to the pathogenesis of autoimmune diseases (Waldmann, 2004). Regarding its metabolic function, it probably participates in the cross-talk between muscle and fat, leading to a reduction of the latter. This was shown experimentally in a rat model, in which IL-15 administration resulted in a 33% loss of white adipose tissue mass (Carbó et al., 2001).

Other important myokines are IL-6 and IL-8, brain-derived neurotrophic factor, leukaemia inhibitory factor, follistatin-like 1 and fibroblast growth factor-21, which are produced by muscle after exercise (Pedersen et al., 2007; Broholm et al., 2008; Izumiya et al., 2008; Pedersen, 2011).

In chronic alcoholism, muscle wasting is a prominent feature. Chronic alcoholic myopathy has been found in nearly 50–60% alcoholics (Peters et al., 1985; Urbano-Márquez et al., 1989; Preedy et al., 2001), and it is defined by muscle atrophy, predominantly affecting type IIb fibres (Preedy and Peters, 1990; Conde-Martel et al., 1992) and leading, sometimes, to incapacitating weakness. To our knowledge, the behaviour of IL-15 in alcoholic patients has not been analysed, nor its relation with muscle wasting, nutritional status or liver function. This may be an important issue, since, as commented earlier, IL-15 administration has proven therapeutic efficacy, antagonizing the muscle breakdown observed in cancer cachexia. So, in this study, we analysed the relationship between serum IL-15 levels and lean and fat mass of different parts of the body, liver function, nutritional status and alcohol intake, in a group of heavy drinkers. In addition, we analysed the behaviour of other cytokines, such as IL-8 and IL-6, also considered as myokines, and of TNF-α, a pro-inflammatory, cachectizing cytokine.

PATIENTS AND METHODS

We included 35 alcoholic patients (four women), consecutively admitted to our hospitalization unit, aged 53.3 ± 11.58 years. All of them were heavy drinkers of >150 g ethanol/day during a prolonged period (>5 years), with an estimated total, lifelong consumption of 24 ± 16 kg ethanol/kg body weight (median = 19; inter-quartile range (IQR) = 12–33). In six cases, antibodies against hepatitis C virus were also detected. Eight patients developed convulsions at admission in the context of withdrawal syndrome. The control group comprised 13 healthy age- and sex-matched sanitary workers (four women), aged 47.0 ± 9.51 years, drinkers of <10 g
Blood samples were taken at 8.00 am in fasting conditions, with patients being initiated by the addition of 25 ml of 0.11 M thiobarbituric acid-reactive substance (TBARS), which was measured according to the method described by Kikugawa (1992). A volume sample of 0.2 ml of plasma was added to 25 µl of 0.11 M thiobarbituric acid, followed by the addition of 0.2 ml of H3PO4 (0.2 M) and the colour reaction was initiated by the addition of 25 µl of 0.11 M thiobarbituric acid.

Liver biopsy was performed when clinically indicated, on 10 patients. Diagnosis of liver cirrhosis was made in 12 cases. Although the Child-Pugh score was designed only for cirrhotic patients, we calculated it for all the 35 alcoholics. Patients were then grouped into those with decompensated cirrhosis (Child B and Child C patients) and those with compensated liver disease (Child A patients). Five patients died during the study period, due to liver failure.

Table 1. Some epidemiological and biochemical variables in patients and controls

<table>
<thead>
<tr>
<th>Patients</th>
<th>Controls</th>
<th>Significance</th>
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<tbody>
<tr>
<td>Age (years)</td>
<td>53.31 ± 11.58</td>
<td>47.00 ± 9.51</td>
</tr>
<tr>
<td>Interleukin-15 (pg/ml)</td>
<td>14.48 ± 44.81; 4.2 (2.98–7.65)</td>
<td>1.69 ± 0.26; 1.80 (1.40–1.90)</td>
</tr>
<tr>
<td>Interleukin-6 (pg/ml)</td>
<td>43.66 ± 54.59; 29.8 (10.6–54.13)</td>
<td>6.46 ± 1.46; 6.80 (5.0–7.55)</td>
</tr>
<tr>
<td>Interleukin-8 (pg/ml)</td>
<td>27.87 ± 46.72; 13.75 (7.57–24.28)</td>
<td>6.05 ± 1.92; 5.0 (5.0–7.0)</td>
</tr>
<tr>
<td>Tumor necrosis factor-alpha (pg/ml)</td>
<td>44.75 ± 169.06; 13.85 (10.40–17.75)</td>
<td>6.25 ± 1.76; 5.60 (5.0–7.45)</td>
</tr>
<tr>
<td>Malondialdehyde (µmol/ml)</td>
<td>3.74 ± 4.05; 2.12 (2.98–7.65)</td>
<td>1.43 ± 0.82; 1.08 (0.73–2.29)</td>
</tr>
<tr>
<td>ASAT (U/l)</td>
<td>77.85 ± 25.04; 50 (30–90)</td>
<td>24.38 ± 6.29; 22 (18.5–30)</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>49.06 ± 39.79; 35 (23–71)</td>
<td>20.46 ± 3.62; 20 (17.5–23.5)</td>
</tr>
<tr>
<td>Sodium (mEq/l)</td>
<td>132.3 ± 9.23</td>
<td>135.2 ± 7.90</td>
</tr>
<tr>
<td>Total leukocyte (pro/mm3)</td>
<td>293.6 ± 504.56; 149 (46.5–252.75)</td>
<td>23.0 ± 9.34; 20 (16–27.5)</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>98.89 ± 8.26</td>
<td>90.0 ± 4.02</td>
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<tr>
<td>LDH (U/l)</td>
<td>292.94 ± 173.54; 251.5 (183.3–343)</td>
<td>0.95 ± 0.12</td>
</tr>
<tr>
<td>CPK (U/l)</td>
<td>1522.4 ± 5779.98; 44.50 (28.0–238.8)</td>
<td>0.76; NS</td>
</tr>
<tr>
<td>Prothrombin activity (%)</td>
<td>97.59 ± 25.04</td>
<td>99.15 ± 2.08</td>
</tr>
<tr>
<td>Serum albumin (g/dl)</td>
<td>3.66 ± 0.75</td>
<td>4.28 ± 0.17</td>
</tr>
<tr>
<td>Serum bilirubin (mg/dl)</td>
<td>2.76 ± 3.65; 1.2 (0.5–3.8)</td>
<td>0.85 ± 0.12; 0.9 (0.8–0.95)</td>
</tr>
<tr>
<td>Total leukocyte (pro/mm³)</td>
<td>7060 ± 3266</td>
<td>Normal 6000–10000</td>
</tr>
<tr>
<td>% Neutrophils</td>
<td>62.32 ± 11.66</td>
<td>60–80%</td>
</tr>
<tr>
<td>% Lymphocytes</td>
<td>23.81 ± 10.67</td>
<td>20–35%</td>
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Values are given as means ± standard deviations, and, in the case of variables with non-parametric distributions, also as median (inter-quartile range). Reference values are given for some commonly used variables, since they were not determined in the control population. When variables showed a parametric distribution, Student’s t-test (T) was used to compare values of patients and controls; in the case of non-parametric distributions, statistical significance was assessed using Mann–Whitney’s U test (Z).

Liver biopsy was performed when clinically indicated, on 10 patients. Diagnosis of liver cirrhosis was made in 12 cases. Although the Child-Pugh score was designed only for cirrhotic patients, we calculated it for all the 35 alcoholics. Patients were then grouped into those with decompensated liver disease (Child B and Child C patients) and those with compensated liver disease (Child A patients). Five patients died during the study period, due to liver failure.

After giving written informed consent, patients and controls underwent an assessment of lean mass and fat mass by dual-energy X-ray absorptiometry with a HOLOGIC QDR-2000 equipment (Waltham, MA, USA), recording both parameters at trunk, right and left arms and right and left legs.

Subjective nutritional assessment included examination of the muscle masses of the upper and lower limbs and of the temporal muscle, defining two degrees of atrophy (severe and moderate) and absence of atrophy. We assigned 2, 1 and 0 points to each category, respectively. We also recorded, by physical examination, fat loss on the cheek and abdomen (Bichat’s fat and subcutaneous fat atrophy) and classified them in a similar way. Each patient was assigned a score, based on the sum of the assigned points, for which the poorest value was 10 and 0 the best (Hernández-Plasencia et al., 1991).

Cytokines and biochemical parameters

Blood samples were taken at 8.00 am in fasting conditions, 2–3 days after hospital admission and were immediately frozen at −80. The following parameters were determined: TNF-α by immunometric chemiluminescent assay (intra-assay variation coefficient ranging 4–6.5%, inter-assay variation coefficient ranging 2.6–3.6%, recovery 92–112%, Diagnostic Products Corporation (DPC), Los Angeles, CA, USA); IL-6, by chemiluminescent assay (intra-assay variation coefficient ranging 5.3–7.5%, recovery = 85–104%, DPC); IL-8, by chemiluminescent assay (intra-assay variation coefficient ranging 5.3–7.5%, DPC); and IL-15 by ELISA (sensitivity <3 pg/ml; range of detection 15.6–1000 pg/ml; Phoenix Pharmaceuticals, Burlingame, CA, USA). In addition, patients underwent routine laboratory tests, including prothrombin activity, serum albumin and bilirubin, aspartate and alanine aminotransferases (ASAT and ALAT), gamma-glutamyltransferase (GGT), lactate dehydrogenase (LDH) and creatine phosphokinase (CPK) in most cases, as well as leucocyte count and mean corpuscular volume (MCV).

Lipid peroxidation products

Serum malondialdehyde (MDA) levels, referred to as thiobarbituric acid-reactive substance (TBARS), were measured according to the method described by Kikugawa et al. (1992). A volume sample of 0.2 ml of plasma was added to 0.2 ml of H2PO4 (0.2 M) and the colour reaction was initiated by the addition of 25 µl of 0.11 M thiobarbituric acid.
acid solution. Samples were placed in a 90°C heating block for 45 min. After the samples were cooled, the TBARS (pink complex colour) were extracted with 0.4 ml of n-butanol. The butanol phase was separated by centrifugation at ×6000 g for 10 min. Aliquots of the n-butanol phase were placed in a 96-well plate and read at 535 nm in a microplate spectrophotometer reader (Benchmark Plus, Bio-Rad, Hercules, CA, USA). The calibration curve was prepared with authentic MDA standards ranging from 0–20 μM. The intra-and inter-assay variation coefficients were 1.82 and 4.01, respectively.

We recorded the presence or absence of ascites, encephalopathy, and calculated the Child-Pugh index, together with prothrombin activity, serum albumin and bilirubin.

The study protocol was approved by the local ethical research committee of our Hospital (2009/23) and conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

Statistics
The Kolmogorov–Smirnov test was used to test normality, a condition not fulfilled by the cytokines analysed. When variables did not fit into a normal distribution, non-parametric tests, such as Mann–Whitney U test and Kruskal–Wallis were used to analyse differences between groups, and Spearman’s correlation coefficient was used to compare quantitative parameters. When variables did show a normal distribution, Student’s t-test, ANOVA, and Pearson’s test were employed.

RESULTS
As shown in Table 1, all the cytokines determined, including IL-15, and MDA levels, were significantly higher in alcoholic patients than in controls. No differences were observed between sexes, or between those with concomitant hepatitis C virus infection and those without it.

All the patients were heavy drinkers (median = 148 IQR = 100–200 g) of many years standing (median = 30 years; IQR = 25–35 years). We found a significant relationship between IL-6 and duration of ethanol consumption (rho = 0.41; P = 0.028) and between IL-8 and daily ethanol consumption (rho = 0.42; P = 0.022) and total lifetime ethanol consumption (in kg ethanol/kg weight, rho = 0.40; P = 0.030). In addition, significant relationships were observed between the ASAT/ALAT index and IL-6 (rho = 0.48; P = 0.005), IL-8 (rho = 0.57; P = 0.001) and MDA (rho = 0.48; P = 0.012).

Only MDA (Z = 2.01; P = 0.045) and IL-6 (Z = 2.51; P = 0.012; Fig. 1a) were significantly higher in cirrhotics, in patients with ascitis (Z = 2.17; P = 0.03 and Z = 3.08, P = 0.002, respectively; Fig. 1b), and in patients with encephalopathy (Z = 2.08; P = 0.038 and Z = 2.38; P = 0.015, respectively; Fig. 1c). Of the biochemical parameters related with liver function, only serum bilirubin showed a direct relation with IL-15 (rho = 0.37; P = 0.047) and with MDA (rho = 0.40; P = 0.042). No differences were observed between patients belonging to Child’s C group and the remaining patients.

All the patients but two were followed-up as outpatients. Within 18 months, 5 of them died (of liver failure). IL-15 (Z = 2.38; P = 0.017; Fig. 2) and IL-6 (Z = 2.18; P = 0.028) were significantly higher among those who died compared with survivors.

No relation was found between any of the cytokines analysed and lean mass at right arm or left arm, except for an inverse correlation between left arm lean mass and IL-6 (rho...
MDA levels (\( \rho = 0.60 \); related with neutrophils proportion (\( \rho = 0.40 \); \( P = 0.007 \)). Cytokines were not related with leukocyte count, but no correlation was observed between body mass index and any of the cytokines or MDA. Also, no correlations were observed between cytokines and MDA and LDH or CPK levels. No differences were observed between patients with or without convulsions at admission for any of the cytokines analysed.

No relations were found between IL-15 and any of the other cytokines analysed. IL-8 was directly related with IL-6 (\( \rho = 0.40; \ P = 0.025 \)), TNF (\( \rho = 0.35; \ P = 0.049 \)) and MDA levels (\( \rho = 0.60; \ P = 0.002 \)). A significant relationship was also observed between TNF and MDA levels (\( \rho = 0.44; \ P = 0.027 \)), and MDA levels were also directly related with neutrophils proportion (\( \rho = 0.40; \ P = 0.048 \)), and, inversely, with lymphocyte proportion (\( \rho = -0.52; \ P = 0.007 \)). Cytokines were not related with leukocyte count, total neutrophils or total lymphocytes.

**DISCUSSION**

The aim of this study was not to analyse if muscle contraction in alcoholics releases more or less amount of cytokines than muscles of control individuals, but to assess which is the behaviour of the so-called myokines in alcoholic patients with varying degrees of liver function impairment, deranged nutrition or reduced muscle mass, admitted to our unit mainly due to organic complications of alcoholism and/or major withdrawal syndrome.

As commented earlier, IL-15 seems to exert anabolic effects, increasing protein content in muscle fibres (Furmanczyk and Quinn, 2003) and promoting myogenic differentiation and muscle growth, with an opposite effect on muscle to that exerted by TNF-\( \alpha \). It is also a potent activator of T and B lymphocytes, neutrophils and NK cells (Fehniger and Caliguri, 2001). In this study, we have failed to find any association between muscle mass and IL-15 levels, which were only directly related with serum bilirubin, pointing to an effect of liver function impairment on its elevation. In this sense, although no differences in IL-15 were observed in relation with the presence or not of advanced liver disease, those who died within the short interval of follow-up showed higher IL-15 values than survivors, supporting the hypothesis that IL-15 acts, like IL-6, as a marker of liver dysfunction or impaired general status.

Although not significantly different, muscle mass in controls were slightly, non-significantly higher than that of patients, but IL-15, like the other cytokines analysed, was significantly lower in controls, strongly speaking against the notion that increased myokine levels in alcoholics are related only to muscle sources. The lack of differences between those with and without convulsions may be also interpreted as indicating that muscle contraction associated with convulsions, at least in alcoholics, does not lead to sustained elevation of myokines; alternatively, it may mean that the effect of muscle contraction on myokine secretion is ‘diluted’ by the production of myokines by tissue other than muscle. In addition, although dynamometry was not assessed in this study, the lack of correlations between IL-15 and convulsions and lean mass is in accordance with an unpublished observation on 20 patients affected by hepatitis C virus infection, 4 of them also alcoholics, in whom no relation was observed—even a trend to a negative one (\( \rho = -0.395; \ P = 0.085 \)—between handgrip strength and IL-15 levels.

IL-15 is probably involved in the cross-talk between muscle and fat, leading to a reduction of the latter. Some studies have shown an inverse correlation between serum IL-15 and trunk fat mass, although not with limb fat mass (Nielsen et al., 2008). In this study, a weak trend towards an inverse relationship between IL-15 and fat mass was observed in alcoholic patients (for instance, \( r = -0.33; \ P = 0.08 \) with left arm fat mass), a trend which became statistically significant when patients and controls were pooled together (\( \rho = -0.43; \ P = 0.009 \) with left arm and \( \rho = -0.42; \ P = 0.011 \) with right arm). These results might be interpreted as a consequence of the lowering effect of this myokine on fat mass. Controls, which showed lower IL-15 levels, had significantly more fat than alcoholics, as shown in Table 2. However, as commented before, the only significant relationships between IL-15 and other parameters observed in alcoholics were the one with serum bilirubin and that with mortality.

Cytokines analysed in this study are not exclusively produced by muscle. This is especially notorious for IL-6, a widely distributed cytokine despite being considered as the prototype of a myokine (Pedersen, 2009). Indeed, we found an inverse relationship between serum IL-6 and left arm muscle mass; we interpret this result in the context of the deranged nutrition and/or more intense muscle atrophy observed in more advanced alcoholic patients, in accordance with more pronounced elevation of IL-6 in patients with ascitis, encephalopathy or liver cirrhosis, and also with the duration of alcohol addiction. Moreover, patients who died showed significantly higher IL-6 levels than patients who

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**Fig. 2.** IL-15 levels are higher in patients who died (all of them due to severe alcoholic hepatitis). In this study, IL-15 behaves similarly to other inflammatory cytokines, such as IL-6.
survived, a result which also suggests a relationship between IL-6 and a more impaired general status. In parallel with IL-6, MDA levels were also related with liver function derangement. Also worthy of note were the relationships observed between MDA and TNF-α and IL-8, as well as between MDA and leukocytes. All these results are consistent with the outstanding role of lipid peroxidation in alcoholic liver disease (Loguercio and Federico, 2003; Cederbaum et al., 2009). However, no relation was observed between MDA and IL-15.

Skeletal muscle is also able to express cytokines such as IL-8, whose levels may be marked raised after exercise. It is also an organ sensitive to the effects of TNF-α. As with the other cytokines studied, the alterations of IL-8 and TNF-α seem to be more dependent on the degree of alcoholism and derangement of liver function and overall nutritional status than on muscle alterations. In the absence of exogenous anabolic stimuli, TNF-α directly provokes a time- and concentration-dependent loss in muscle specific proteins, including myosin heavy chain (Li et al., 1998). However, in this study, we also failed to find any relation between muscle wasting and TNF-α levels.

We therefore conclude that in alcoholics, IL-15 levels are higher than in controls, and even more so among those who died within 18 months after admission. They are not related with muscle mass, degree of alcoholism or nutritional status, but only with serum bilirubin. IL-6, another myokine, showed inverse correlations with liver function, degree of alcoholism, nutritional status and left arm muscle mass, as well as with short-term mortality.

REFERENCES


