α_2 -Macroglobulin expression in the liver in response to inflammation is mediated by the testis

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Abstract

Earlier studies have shown that germ cells or germ cell-conditioned media are capable of regulating α_2 -macroglobulin (α_2 -MG, a non-specific protease inhibitor) expression by Sertoli cells and hepatocytes cultured *in vitro*. These results illustrate a possible physiological link between testes and liver regarding α_2 -MG production. Using a series of surgical procedures including castration, hemicastration, and hepatectomy coupled with Northern blot and immunoblot analyses, we report herein that the surge in α_2 -MG expression in the liver in response to inflammation is indeed regulated, at least in part, by the testis via testosterone. It was found that hepatectomy induced at least a tenfold increase in the steady-state mRNA and protein production of α_2 -MG in the liver. However, castration induced a mild but not statistically significant induction of α_2 -MG in the liver in contrast to sham operation or hemicastration alone, when hemicastration alone could induce liver α_2 -MG production by almost fourfold. Perhaps most important of all, hepatectomy

accompanied by castration significantly reduced the liver α_2 -MG response to the surgery-induced inflammation compared with hepatectomy alone, illustrating that the removal of the testicles can induce a loss of signal communications between the testis and the liver, rendering a significant loss of the α_2 -MG response to experimentally induced inflammation in the liver. Interestingly, this lack of response of the liver to surgery-induced inflammation regarding α_2 -MG production following castration could be restored, at least in part, by using testosterone implants placed subdermally 6 days prior to orchietomy. Collectively, these results illustrate that a physiological link does indeed exist between the testis and the liver, and that testes *per se* can influence the liver α_2 -MG expression in response to inflammation *in vivo* possibly via testosterone or testosterone-induced biological factor(s).

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Introduction

α_2 -Macroglobulin (α_2 -MG) is a 720 kDa glycoprotein consisting of four identical subunits of ~180 kDa for each subunit. It functions as a non-specific protease inhibitor in mammals which is predominantly produced in the liver, and it is one of the major acute-phase serum proteins associated with inflammatory response (Kushner 1982). Serum α_2 -MG is also capable of binding to a broad spectrum of cytokines, such as transforming growth factor (Huang *et al.* 1988) and basic fibroblast growth factor (Dennis *et al.* 1989). This α_2 -MG–cytokine complex formation is believed to provide a mechanism to regulate the bioavailability of cytokines and to clear excessive cytokines during inflammation and wound repair (Gaddy-Kurten *et al.* 1989). Apart from the liver, other tissues such

as ovary, uterus, placenta and testes have also been shown to synthesize α_2 -MG. For instance, Sertoli cells are the source of α_2 -MG in the rat testis (Cheng *et al.* 1990), and α_2 -MG in the testis can bind to TGF- β 3 (Wang *et al.* 2004). Unlike the liver, testicular α_2 -MG is not induced in response to inflammation, such as during experimental inflammation following administration of fermented yeast *in vivo* (Li *et al.* 1994). Interestingly, the steady-state mRNA or the protein level of α_2 -MG in the testis is several orders of magnitude higher than in the liver (Li *et al.* 1994), illustrating that its level must be maintained at a relatively high level for spermatogenesis. Indeed, recent studies have shown that α_2 -MG in the testis may be used to prevent unchecked proteolytic activity induced by degenerating germ cells, implicating its involvement in tissue remodeling pertinent to spermatogenesis in the

seminiferous epithelium (Zhu *et al.* 1994). Additionally, α_2 -MG is apparently being used to protect the testis from the assaults of environmental toxicants, such as cadmium toxicity, via the c-Jun N-terminal protein kinase signaling pathway (Wong *et al.* 2004, 2005). Even though hepatocytes and Sertoli cells both synthesize and secrete α_2 -MG, its production by Sertoli cells, unlike hepatocytes, does not respond to interleukin-6 treatment *in vitro*, confirming that α_2 -MG is not an acute phase protein in the testis (Zwain *et al.* 1993). Yet α_2 -MG production by Sertoli cells could be stimulated by germ cells and germ cell-conditioned medium (GCCM) (Braghiroli *et al.* 1998), which seemingly suggests that germ cells may release a soluble factor(s) to regulate Sertoli cell α_2 -MG expression. Interestingly, GCCM was also shown to stimulate hepatocyte α_2 -MG production *in vitro* in this earlier study (Braghiroli *et al.* 1998). However, a physiological link between the testis and the liver *in vivo* is not known. Although there is no visible anatomical linkage between testis and liver, it is plausible that germ cells secrete soluble factors apically to regulate Sertoli cells as well as releasing these factors basally to regulate hepatocyte α_2 -MG via the systemic circulation. In this study, certain surgical operations, such as hepatectomy and castration, were performed in adult rats to assess whether a physiological link exists between these two organs *in vivo*.

Materials and Methods

Animals

Male Sprague-Dawley rats at 60 days of age were obtained from Charles River Laboratories (Kingston, New York, USA). Rats in groups of three at specific time points for a particular surgery were killed following treatment and/or surgery by CO₂ asphyxiation. Testes and livers were removed immediately and kept at -80°C until used. The use of animals for these studies was approved by the Rockefeller University Animal Care and Use Committee with protocol numbers 00111, 95129-R and 03017.

Partial hepatectomy

Adult rats ranging between 380 and 400 g body weight (bw) were anesthetized with ketamine HCl (Ketaset, Fort Dodge Animal Health, Fort Dodge, IA, USA) at 50–60 mg/kg bw administered intramuscularly (i.m.) near the thigh using a 26-gauge needle. Hair at the surgical site was removed using an electric shaver, and the area was carefully cleansed with 70% ethanol and Betadine (twice each). A small incision, ~ 2.5 –3 cm, was made using a sterile surgical blade at the surgical site to expose the liver, and two-thirds of the liver was removed as described earlier (Higgins & Anderson 1931). After bleeding stopped, the abdomen was closed using sterile nylon black

monofilament suture (Ethicon Inc., Somerville, NJ, USA), and rats were allowed to recover. Sham operations were carried out via an abdominal incision (~ 2.5 cm long) with manipulation of the liver lobes instead of an incision in the liver. Animals were killed at 6 h, 24 h, 48 h, 72 h, 1 week and 4 weeks after operation. Liver and testes were removed immediately, frozen in liquid nitrogen, and stored at -80°C until used for RNA extraction or protein lysates preparation.

Castration and hemicastration

Animals were anesthetized with Metofane (2,2-dichloro-1,1-difluoro-ethyl methyl ether; Mallinckrodt Veterinary Inc., Mundelein, IL, USA). The scrotum was cleansed with 70% ethanol and Betadine (twice each). A small incision (~ 1 cm) was made to expose the testis. The testicular vein and artery were tied with sterile and absorbable surgical suture (Ethicon 3-0 chromic gut suture) above the testis to minimize blood loss. Thereafter, one (hemicastration) or both (castration) testes were removed. Sham operations were performed by making a scrotum incision of ~ 1 cm without the removal of the testis. Thereafter, the surgical site was cleansed with 70% ethanol, and stitched with nylon black monofilament suture, and the rats were allowed to recover. Thereafter, animals were killed at 6 h, 24 h, 48 h, 72 h, 1 week and 4 weeks by CO₂ asphyxiation. Liver and/or testis were removed, frozen in liquid nitrogen immediately, and stored at -80°C until used for RNA extraction and/or lysate preparation.

Preparation of steroid implants and their placement subdermally in adult rats

To assess if testosterone replacement could restore the inflammatory response in the liver regarding α_2 -MG production after orchiectomy, the following experiment was performed. Testosterone implants were prepared and their subdermal placement in adult rats (~ 400 g bw) was performed as described by Zhang *et al.* (2005). In brief, 4-cm implants were prepared just before surgery by filling ethylene and vinyl acetate (EVA) tubing (Elvax 770, 9% VA; 2.15 mm i.d. $\times$ 2.4 mm o.d.; Du Pont, Wilmington, DE, USA) with testosterone (Sigma); both ends were sealed by heat using a soldering iron, and cleansed with 70% ethanol prior to their use. Earlier studies have shown that implants using EVA had a steroid release rate (e.g. of 7 α -methyl-19-nortestosterone) of ~ 90 $\mu\text{g}/\text{cm}^2/\text{day}$ *in vivo* when placed under the skin in humans (Noe *et al.* 1999, von Eckardstein *et al.* 2003). Animal surgery was performed in adult rats (~ 400 g bw) under anesthesia using ketamine HCl at 60 mg/kg bw (i.m.). Hair at the surgical site on the dorsal side of the rat was removed, and the skin cleansed with 70% ethanol and Betadine (twice each). A small incision was immediately opened (~ 2 cm), and four

4-cm testosterone implants were inserted subdermally. The surgical site was then cleansed with 70% ethanol, and stitched with nylon black monofilament suture. Rats were allowed to recover for 6 days until the level of α_2 -MG subsided (see Fig. 1). On day 7, castration was performed with both testes removed as described above. Rats were killed at 6 and 24 h thereafter, and livers were removed and stored at -80°C until used for protein lysate preparation. Because the testosterone implants sustained the endogenous testosterone levels, the loss of androgens in the systemic circulation following orchiectomy was negligible.

RNA extraction and Northern analysis

Total RNA was extracted from liver and testis using RNA STAT-60 (Tel-Test Inc., Friendswood, TX, USA) as described by Braghiroli *et al.* (1998). Northern blot analysis was performed as previously described using an α - ^{32}P -labeled α_2 -MG cDNA probe by nick translation for hybridization (Braghiroli *et al.* 1998). To ensure that equal amounts of RNA were loaded into each lane within an experimental group, agarose gels were stained with ethidium bromide. In experiments reported in Fig. 1, the blots were stripped and rehybridized with an α - ^{32}P -labeled S-16 cDNA probe, and data were normalized after densitometric scanning analysis to correct for possible uneven loading.

Preparation of liver lysates

Liver was lysed in SDS lysis buffer (0.125 M Tris, pH 6.8, 22 $^\circ\text{C}$, containing 1% SDS, 1.6% 2-mercaptoethanol, 2 mM PMSF, 1 mM EDTA). Samples were homogenized using a Polytron or sonicated using a sonicator (15 s, 2 times, interspaced by 30 s on ice), vortexed, and centrifuged at 15 000 g for 10 min at 4 $^\circ\text{C}$. Supernatants were collected and used as liver lysates. Protein estimation was performed by the Coomassie blue-dye binding assay (Bradford 1976) using BSA as a standard.

Immunoblotting analysis

Protein, 50 μg per sample, was resolved onto 7.5% T SDS-polyacrylamide gels by SDS-PAGE under reducing conditions as described by Cheng and Bardin (1987). Following electrophoresis, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell Inc, Keene, NH, USA). Blots were probed with an α_2 -MG antibody which was characterized earlier (Cheng *et al.* 1990, Stahler *et al.* 1991), followed by bovine anti-rabbit IgG conjugated to horseradish peroxidase and developed using ECL chemiluminescent kits from Amersham-Pharmacia-Biotec as described (Lui *et al.* 2003, 2005).

Results

Changes in α_2 -MG steady-state mRNA level in the liver in response to experimental inflammation

In this study, hepatectomy or castration was used to induce an inflammatory response. It was noted that the basal steady-state mRNA level of α_2 -MG, an acute-phase protein, was barely detectable in the liver of normal rats. However, its mRNA level was induced considerably in response to inflammation as early as 6 h after surgery (Fig. 1A–D), and this 6-h time point was then used as the baseline for densitometric scanning analysis. As such, the fold stimulation of α_2 -MG in response to hepatectomy as reported herein is an underestimate of the actual increase. The steady-state mRNA level of α_2 -MG 24 h after hepatectomy was induced by at least 12-fold over the level at 6 h (Fig. 1A, D). However, castration alone had a much milder effect on the liver α_2 -MG levels compared with hepatectomy (Fig. 1B, D vs 1A, D). For instance, if the increase in α_2 -MG levels in the liver by 6 h post operation was used as the baseline, castration *per se* failed to induce any changes in the liver α_2 -MG level (Fig. 1B, D). More importantly, when castration was performed prior to hepatectomy with a lapse of about 15–50 min, liver α_2 -MG mRNA levels were stimulated by only 7-fold compared with 12-fold for hepatectomy alone (Fig. 1C, D vs 1A, D), illustrating a significant loss of response to hepatectomy-induced inflammation when both testicles were removed, and implying that the testes were releasing a substance(s) that modulates the liver acute-phase response.

Changes in testicular α_2 -MG steady-state mRNA levels in response to inflammation

In this study, the steady-state α_2 -MG mRNA levels in the testis after hepatectomy and hemicastration were also examined and compared with two sham operation procedures. However, we failed to detect any significant changes in testicular α_2 -MG mRNA levels after hepatectomy (Fig. 2A, E) or hemicastration (Fig. 2B, E) when compared with the corresponding controls (Fig. 2C, D and E), suggesting that α_2 -MG is not an acute-phase protein in the testis, consistent with the results of an earlier report (Stahler *et al.* 1991). Furthermore, in contrast to the liver, the basal α_2 -MG level in the testis is significantly higher than in the liver, suggesting that this protein is apparently being activated in the testis for normal functioning pertinent to spermatogenesis.

The testis is crucial in the regulation of liver α_2 -MG expression in response to inflammation

To further confirm that the testis does indeed play a significant physiological role in regulating liver α_2 -MG

Figure 1 Changes in the α_2 -MG steady-state mRNA levels in the liver in response to different surgical procedures. (A-C) Northern blots (left panel) illustrating changes in the steady-state α_2 -MG mRNA levels in the liver after (A) hepatectomy by removing 2/3 of the liver, (B) castration and (C) hepatectomy plus castration. The right panel shows the corresponding ethidium bromide-stained gels of the left panel, illustrating similar amounts of total RNA (15 μ g total RNA per lane) were used for this experiment. (D) The corresponding densitometrically scanned results using autoradiograms such as those shown in A-C are shown. Each bar represents the mean $\pm$ S.D. of samples from three adult Sprague-Dawley rats (~ 300 g bw). Since the basal level of α_2 -MG in the normal liver is virtually undetectable, the level at 6 h after operation was arbitrarily set at 1, against which the increase in α_2 -MG levels at other time points was compared. ns, not significantly different from control (6 h), * $P < 0.05$, ** $P < 0.01$ (by Student's *t*-test). [^] $P < 0.05$ (by ANOVA), comparing data in rats with hepatectomy and castration vs rats with hepatectomy only and rats with castration. h, hour; wk, week; n.d., not detectable.

levels during inflammation, liver α_2 -MG steady-state protein levels were quantified following hemicastration and were compared with the levels following other surgical procedures. Similar to the data shown in Fig. 1, the α_2 -MG protein level was significantly induced in the liver 24 h after hepatectomy (Fig. 3A, E). Interestingly, it was found that hemicastration could stimulate a significant increase in liver α_2 -MG protein levels similar to the sham

operation (scrotum incision) by 24 h after operation (Fig. 3B, C, E). Yet no significant increase in liver α_2 -MG protein levels was detected by 24 h in rats when both testicles were removed (Fig. 3D, E). These results suggest that there is a possible physiological link between the liver and the testis, and that this linkage is important in maintaining the inflammatory response in the liver regarding its α_2 -MG production.

Figure 2 Changes in the α_2 -MG steady-state mRNA levels in testes in response to different surgical procedures. (A-D) Northern blots (left panel) illustrating α_2 -MG expression in testes after (A) hepatectomy, (B) hemicastration, (C) sham operation (abdomen incision) and (D) sham operation (scrotum incision). The right panel shows the corresponding ethidium bromide-stained gels of the left panel, illustrating that similar amounts of total RNA (15 μ g total RNA per lane) were used for this experiment. (E) Histogram showing the corresponding densitometrically scanned results using autoradiograms such as those shown in A-D. Each bar is the mean $\pm$ s.d. of samples from three rats. ns, not significantly different from control (6 h) (by Student's *t*-test). h, hour; wk, week.

The reduced α_2 -MG production in the liver in response to castration-induced inflammation can be restored by using testosterone implants

To assess if the loss of response in the liver regarding α_2 -MG production during surgery-induced inflammation

by removing both testicles (total castration) is mediated by testosterone, the following study was conducted. In order to replace the loss of testosterone following orchietomy, four 4-cm testosterone implants were placed subdermally on the dorsal side of adult rats 6 days prior to castration which was performed on day 7. In this way, the

Figure 3 Changes in liver α_2 -MG protein level in response to different surgical procedures in rats with and without testes, or with one of the two testes. (A-D) Immunoblots (left panel) illustrating the steady-state α_2 -MG protein levels in liver after (A) hepatectomy, (B) sham operation (scrotum incision), (C) hemicastration and (D) total castration. The right panel shows the corresponding blots stained for actin using an anti-actin antibody, illustrating equal protein loading between samples. (E) Histogram showing the corresponding densitometrically scanned results using chemiluminograms such as those shown in A-D. The level of α_2 -MG in the liver at 6 h was arbitrarily set at 1 since the basal level of α_2 -MG in normal liver was virtually undetectable (see Fig. 1). Results are expressed as the mean $\pm$ s.d. of three separate experiments, using samples from different rats. ns, not significantly different from control (6 h); * P <0.01 (by Student's t -test). h, hour.

endogenous testosterone level can be maintained after orchiectomy. Following different surgical procedures (either hepatectomy or castration with and without four 4-cm testosterone implants), rats ($n=3$ per time point) were killed after 6 h or 24 h and two-thirds of the liver was removed for lysate preparation. As expected, the endogenous α_2 -MG steady-state protein levels in the liver were induced by $\sim$ ninefold following hepatectomy (Fig. 4A, D). Likewise, total castration (without testosterone implants) rendered the rats non-responsive to surgery-induced inflammation regarding α_2 -MG production (Fig. 4B, D vs Fig. 4A, D). Yet the presence of four 4-cm testosterone implants could restore, at least in part, the

liver response to surgery-induced inflammation (Fig. 4C, D vs 4B, D), illustrating that androgen is one of the crucial physiological links between the testis and the liver.

Discussion

In this study, we have demonstrated that α_2 -MG is an acute-phase protein in the liver, but not in the testis, which is consistent with our previous observations (Braghiroli *et al.* 1998, Stahler *et al.* 1991). For instance, the liver α_2 -MG mRNA levels increased dramatically by 24 h after hepatectomy, yet no such increase was detected

Figure 4 A study to assess the use of testosterone implants to restore the liver responsiveness to castration-induced α_2 -MG production during inflammation. (A-C) Immunoblots (left panel) illustrating the steady-state α_2 -MG levels in the liver at 6 and 24 h following (A) hepatectomy, (B) total castration without any testosterone implants and (C) total castration in rats in which four 4-cm testosterone implants were placed subdermally for 6 days prior to orchietomy (on day 7) to maintain the endogenous testosterone level. Each time point had 3 adult rats. The right panel shows the same blots as the left panel after these blots were stripped and re-probed with an anti-actin antibody to assess equal protein loading and uniform protein transfer from gels to nitrocellulose membranes. (D) Histogram summarizing the densitometrically scanned results using chemiluminograms such as those shown in A-C and normalized against actin. The level of α_2 -MG in the liver at 6 h was arbitrarily set at 1 since the basal level of α_2 -MG at the time of orchietomy (time 0) was virtually non-detectable (see Fig. 1). Results are expressed as the mean $\pm$ S.D. of 3 rats. ns, not significantly different from the 6 h time point; * P < 0.01 (by Student's t -test). [^] P < 0.01 (by ANOVA), comparing data in rats with testosterone implants and rats without implants.

in the testis after any surgical procedures. However, the basal α_2 -MG level in the testis is significantly higher than that in the liver, and is possibly being used to maintain spermatogenesis in the seminiferous epithelium, for example by protecting the seminiferous epithelium from the unwanted proteolysis associated with germ cell development during the epithelial cycle. Indeed, recent studies have shown that the testis is utilizing proteolysis to facilitate junction restructuring in the seminiferous epithelium pertinent to germ cell migration during spermatogenesis (Mruk *et al.* 1997, Longin *et al.* 2001, Siu & Cheng 2004, Wong *et al.* 2004, 2005) in much the same way as cell adhesion complexes are used to facilitate cell movement at the cell-matrix interface (for reviews, see McCawley & Matrisian 2001, Mruk & Cheng 2004).

Interestingly, the liver α_2 -MG levels were induced more significantly after sham operation (e.g. scrotum incision) than after total castration. While the general inflammatory response caused by total castration is more severe than that caused by sham operation, the inflammatory response of the liver is significantly lower when both testicles have been removed than after sham operation or hemicastration. These results have provided a strong argument for the fact that the testis may be releasing a hormonal substance(s) that can modulate liver α_2 -MG levels in response to inflammation induced by the surgical procedures. It is possible that the testis secretes biological factor(s) into the systemic circulation, which, in turn, accelerates the liver inflammatory response. When both testes are removed in total castration, they can no longer synthesize and secrete these biological factors, resulting in a significantly lowered inflammatory response. This observation also explains our earlier studies that germ cells and GCCM can upregulate α_2 -MG levels in hepatocyte cultures. Since the testis is the principal source of androgens in mammals, we next sought to investigate if the endogenous testosterone level that can be maintained by using testosterone implants when both testicles are removed can restore the liver's responsiveness to castration-induced inflammation regarding its α_2 -MG production. Indeed, the presence of testosterone implants can significantly revive the inflammation-induced α_2 -MG production in the liver following orchiectomy, and this strongly suggests that testosterone is the crucial regulator released from the testis that can regulate acute-phase response in the liver.

While these findings are preliminary in nature regarding the presence of a physiological relationship between the liver and the testis, they are significant in many ways. For instance, they provide proof that there is physiological cross-talk between the liver and the testis via androgens, at least in terms of the acute-phase response in the liver. Furthermore, these results illustrate that androgens are crucial regulators of acute phase response in the liver. It is also likely that testosterone is one of the factors that maintain a high level of α_2 -MG in the seminiferous

epithelium to support spermatogenesis. Nonetheless, there are many questions that remain to be addressed. For example: are these yet-to-be identified testis factors (e.g. testosterone) that can modulate the inflammatory response in the liver, limited only to regulating the level of α_2 -MG? Do these factors regulate other liver functions *in vivo* under normal physiological conditions in addition to the protein secretory function such as α_2 -MG? These questions must be carefully addressed in future studies. These future studies will also shed more light on the physiological link between the testis and the liver.

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