Octreotide inhibits liver regeneration by suppressing regional estrogen receptor type a expression

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Abstract
Background and rationale: Liver regeneration involves a significant variety of growth and paracrine factors. Octreotide has long been shown to inhibit liver regeneration, although the exact mechanism of its action remains unclear. This paper aims to examine the effect of long-term octreotide administration on the expression of the estrogen receptor type alpha (Era) as a potential novel pathway via which liver regeneration may be hindered. Sixty adult male Wistar rats were submitted to 70% (extensive) hepatectomy and subsequently randomized to receive either a subcutaneous injection of 50gr ams/kg body weight octreotide diluted in 1mL of 0.9% normal saline (SS group) or simply 1mL of 0.9% normal saline (NS group). Animals were followed up to 168 or 1440h (1 week and 1 month, respectively) post-hepatectomy and subsequently sacrificed. Removed livers were weighted, diluted in paraformaldehyde, embedded in paraffin wax, sliced at 5 micrometer intervals and prepared for the immunohistochemical detection of ERa. The control group labeling indices for both hepatocytes and cholangiocytes at 168 and 1440h were higher at a statistically significant degree compared to age-matched SS group animals. Interestingly, ERa expression is significantly increased over time in control animals for both cell types examined, while this is not true for animals receiving octreotide. In conclusion, octreotide-mediated inhibition of liver regeneration involves the long-term down-regulation of ERa expression in hepatocytes and cholangiocytes. This hormonal cross-talk may be of particular significance to explain sex-specific differences in liver repair dynamics.

In conclusion
Octreotide inhibits liver regeneration by suppressing regional estrogen receptor type a expression


Introduction
Sex steroids constitute a highly conserved category of hormones involved in a variety of developmental, reproductive and metabolic functions. Via their nuclear and membrane receptors they have been shown to result in a number of effects to various cell functions, including alterations in cell proliferation dynamics and selective tissue differentiation, which are both ultimately associated with both a direct response to environmental signals and a long-term adaptation to stress [1]. These variations in sex steroid expression and action may be potentially of clinical significance, since in a number of pathological settings it has been verified that distorted sex steroid function is associated with increased disease severity or frequency [1-2]. Interestingly, this association doesn’t only refer to reproductive organ disorders, but also extends to other systems, including the liver and biliary tree [3-5].

In the latter case, sexual dimorphism refers to a wide range of sex-specific characteristics, spanning from embryogenesis to normal adult anatomy and physiology [6]. Moreover, there is considerable variance in liver reaction to damage and disease (sex-specific hepatotoxicity), a phenomenon which has been well documented in a number of clinical conditions involving chronic liver infection/hepatitis, such as alcoholic liver disease and viral hepatitis [6-8]. Naturally, a proportion of the clinically observed variance in sex-specific frequency and severity of liver disease may in fact depict differentiation in non organic, external socioeconomic and cultural elements (such as living conditions, occupation, exposure to heavy metals and toxins, nutritional and alcohol consumption habits, medication, sexual practices, substance use etc) [8-10]. Nevertheless, in a number of cases, such as alcoholic liver disease and hepatocellular carcinoma), it has also been possible to demonstrate a sexually dimorphic, differential expression pattern of sex steroid receptors (especially alcoholic liver disease) as a constant molecular finding involved in the pathophysiology of the disease [11, 12].

Being that liver regeneration is by default an adaptive reaction to some form of extrinsic or intrinsic stress (microbial, pharmaceutical, toxic, traumatic, hemodynamic, mechanical or other) it is reasonable to assume that any sex-specific differences observed in this process may also involve sex steroid receptor manipulations [6, 8, 13]. Indeed, via their anabolic effect on metabolism, sex steroids may enhance tissue repair and cell proliferation, thus retaining a positive role in liver regeneration [1, 6]. However, despite evidence that estrogen receptors are indeed differentially expressed in the process and may also be affected by the concomitant circulation of other hormones, such as somatostatin [14-17], it has not so far been attempted to examine their long-term expression dynamics in this context.

The aim of this study is to provide novel data on this subject, by estimating the expression of estrogen receptor type alpha (ERa) one week and one month post partial hepatectomy in Wistar rats and comparing it to animals receiving somatostatin.
Materials and methods

Animal population - Bioethics clearance
All experiments were carried out on male Wistar rats from the colony of the Pasteur Institute, Athens, approximately 8-12 weeks old and with a mean body weight of 250g. All animals were kept under stable conditions (21 degrees Celsius room temperature, 12h light-darkness daily cycle) and had free access to food and tap water until 12h before the operation. All have been conducted in accordance with the recommendations and guidelines of the local University Bioethics Committee, which has also granted permission to proceed with this research protocol.

Partial hepatectomy
All surgeries were carried out between 8-11pm. The operation performed was classic 70% partial hepatectomy [18]. A total of 68 animals were used for the experiment. Five rats were sacrificed immediately after the operation, in order to obtain baseline reference values and three died due to complications. The remaining 60 animals were randomized to receive either 50mg/kg body weight octreotide in 1mL normal saline (SS group) or 1mL normal saline (NS group) subcutaneously every 12h. The animals were sacrificed at 168 and 1440h (1 week and 1 month, respectively) post-operatively, thus forming 2 age subgroups per group, each consisting of 15 animals. In all cases, the liver was removed and weighted.

Histology
Liver tissue samples were fixed in formalin for 12h, processed routinely, and embedded in paraffin wax. Sections were cut via microtome to form serial slices of 3 micrometer thickness. One in every ten such slides was stained with hematoxylin and eosin to detect the major histological characteristics in the dissected liver (i.e. active regeneration, inflammation-necrosis and normal liver areas). The remaining slides were used for immunohistochemistry (Fig. 1, 2).

Figure 1. PCNA labeling in the regenerating rat liver. A) 168h post-hepatectomy, control group B) 168h post-hepatectomy, octreotide group C) 1440h post-hepatectomy, control group D) 1440h post-hepatectomy, octreotide group. Counterstaining via hematoxylin, magnification * 100.

Immunohistochemistry
For the estimation of liver proliferation, the labeling index of the proliferating cell nuclear antigen PCNA (polyclonal anti rabbit primary antibody-DAKO) was immunohistochemically calculated, using a dilution of 1:40 [17, 18]. For the estimation of ERa (estrogen receptor a) in hepatocytes and cholangiocytes, the labeling index of ERa (Santa Cruz polyclonal anti-rabbit primary antibody) was calculated, using the manufacturer proposed dilution of 1:50. For the detection of the antigens in paraffin sections of rat tissues, antigen retrieval is necessary. Therefore, after dewaxing (via dry heat, scaled hydration and exposure to phosphate buffer saline-PBS), sections were immersed in 10mmol/L citric acid, pH 6, and boiled for 3min in a microwave oven. Subsequently slides were rinsed with PBS and endogenous peroxidase was blocked by incubating the slides in 0.2% H2O2 for 20min. Sections were then processed with blocking serum (S-2000 blocking serum VECTOR) for 30min, followed by 1-hour incubation with the primary antibody. Sections were subsequently incubated with the secondary antibody for 45min, followed by exposure to the Avidin/Biotin Complex for 30min. Finally, the reaction was visualized after exposure to a diaminobenzidine solution for 10min. Slides were counter-stained with hematoxylin. The labeling index for each antigen was the percentage of positive to total stained nuclei. Quantitative analysis was performed using a Leica Microsystems photon microscope and Image Pro Plus.
Figure 2. ERα labeling in hepatocytes and cholangiocytes in the regenerating rat liver. A) 168h post-hepatectomy, control group B) 168h post-hepatectomy, octreotide group C) 1440h post-hepatectomy, control group D) 1440h post-hepatectomy, octreotide group. Counterstaining via hematoxylin, magnification * 100.

Statistical analysis
Statistical analysis was performed using SPSS 18.0 (Statistical Package for Social Sciences, Chicago). Values were expressed as mean±SD and compared with the Student’s t-test. The minimum level of statistical significance was set at 95% (P<0.05).

Results
Weights of the removed liver following sacrifice were measured per age group. Results were calculated as a means of all 15 animals per age. The means were then compared using the student’s t-test assuming a statistical significance level of 95%. A summary of outcomes is presented in Table 1.

Table 1. Comparison of mean weight of removed liver per age and group. For both 168 and 1440h, liver weight was higher in the control group at a statistically significant level.

<table>
<thead>
<tr>
<th></th>
<th>NS-mean</th>
<th>NS-SD</th>
<th>SS-mean</th>
<th>SS-SD</th>
<th>T-test</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>168h</td>
<td>6.4</td>
<td>0.6</td>
<td>4.8</td>
<td>1.2</td>
<td>4.62</td>
<td>&lt;0.001</td>
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<tr>
<td>1440h</td>
<td>11.38</td>
<td>0.35</td>
<td>11.09</td>
<td>0.34</td>
<td>2.30</td>
<td>0.03</td>
</tr>
</tbody>
</table>

The PCNA labeling index in hepatocytes has been measured as an indicator of cell proliferation potential. Although the control group presents with higher estimations for both age groups tested, the difference was only proven to be statistically significant 1 month after hepatectomy. A summary of results is shown in Table 2.

Table 2. Comparison of PCNA labeling indexes in the hepatocytes of the removed liver per age and group. For 1440h, ERα expression across time exhibits a positive tendency, with a statistically significant increase between 168 and 1440h for both hepatocytes and cholangiocytes. In octreotide-treated animals, the normal ERα expression curve is reversed in hepatocytes, with a decrease in overall expression between 168 and 1440h. No measurable difference has been noted for cholangiocytes.

In control animals, ERα expression across time exhibits a positive tendency, with a statistically significant increase between 168 and 1440h for both hepatocytes and cholangiocytes. In octreotide-treated animals, the normal ERα expression curve is reversed in hepatocytes, with a decrease in overall expression between 168 and 1440h. No measurable difference has been noted for cholangiocytes.
**PCNA expression in hepatocytes was higher in the control group at a statistically significant level**

<table>
<thead>
<tr>
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<th>NS-mean</th>
<th>NS-SD</th>
<th>SS-mean</th>
<th>SS-SD</th>
<th>T-test</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>168h</td>
<td>0.19</td>
<td>0.1</td>
<td>0.15</td>
<td>0.06</td>
<td>1.33</td>
<td>NS</td>
</tr>
<tr>
<td>1440h</td>
<td>0.36</td>
<td>0.14</td>
<td>0.27</td>
<td>0.08</td>
<td>2.16</td>
<td>0.048</td>
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</tbody>
</table>

**Table 3. Hepatocyte and cholangiocyte ERα labeling indexes at 168h post-hepatectomy. For both cell populations, the labeling in the control group exceeded that of the octreotide group at a statistically significant level.**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>NS-mean</th>
<th>NS-SD</th>
<th>SS-mean</th>
<th>SS-SD</th>
<th>T-test</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholangiocytes</td>
<td>62.7</td>
<td>0.83</td>
<td>28</td>
<td>17.8</td>
<td>6.75</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>4.4</td>
<td>3.95</td>
<td>1.47</td>
<td>1.55</td>
<td>2.39</td>
<td>0.03</td>
</tr>
</tbody>
</table>

**Table 4. Hepatocyte and cholangiocyte ERα labeling indexes at 1440h post partial hepatectomy. For both cell populations, the labeling in the control group exceeded that of the octreotide group at a statistically significant level (P<0.001).**

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>NS-mean</th>
<th>NS-SD</th>
<th>SS-mean</th>
<th>SS-SD</th>
<th>T-test</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholangiocytes</td>
<td>72.97</td>
<td>7.01</td>
<td>17.42</td>
<td>8</td>
<td>18.09</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>57.96</td>
<td>32.17</td>
<td>2.16</td>
<td>0.89</td>
<td>6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

**Discussion**

The comparison of anatomical, histological and immunohistochemical findings between control and octreotide-treated animals have revealed considerable differences in the regenerative dynamics of the rat liver. These findings verify the inhibitory role of octreotide in liver regeneration, as it has been demonstrated in classical studies over the last 30 years but also indicate a so far unidentified direct implication of estrogen receptor alpha and, therefore, sex steroids, in this mechanism [14-17].

In particular, at a macroscopic level, both 168 and 1440h post-hepatectomy, the octreotide-treated group exhibited a smaller removed liver weight compared to age-matched controls. In relative terms, one might mention that a week/168h post-hepatectomy, regeneration rate in control animals reached about 60% of expected total average liver weight, whereas the same proportional allocation for the octreotide-treated group was reduced to less than 40%. The capacity of somatostatin to inhibit liver regeneration in the selected experimental conditions was particularly high, with an almost 50% reduction rate over the first week post partial hepatectomy. This finding has been calculated by the fact that mean remnant liver tissue post-hepatectomy is estimated at approximately 30% of original mass and therefore, control group animals have in fact doubled their liver mass by the end of the first week, contrary to the octreotide-treated animals, where liver mass was only increased by about a third. Extending these observations to the end of the study period, one might mention that liver regeneration is extensive in both groups (over 95% of original mass regained), but still, a measurable statistically significance remains in favor of the control group. These findings are largely in accordance with those available for early and mid-term follow up of rat liver regeneration after octreotide administration, proving that its inhibitory effect may be both prolonged and sustained [14, 19-21].

The histological features of distorted liver regeneration in animals submitted to octreotide administration, compared to age matched controls are well correlated to those reported in previous publications in the field [14, 16, 20]. These include, among others, a larger proportion of apoptotic hepatocytes, a smaller number of mitotic divisions for both hepatocytes and cholangiocytes and a reduction in overall regenerative kinetics both in terms of cholangiolar hyperplasia and oval cell proliferation and distribution, resulting in delayed portal triad and ultimately liver lobe formation in the intervention group [19-21].

With regard to cell proliferation dynamics, the study has the nuclear antigen PCNA as an indicator of the proportion of cells remaining in the active steps of the cell cycle, contrary to the resting population in G0 state [22, 23]. The qualitative and quantitative study of PCNA expression in hepatocytes in our study has revealed a constant superiority of the control group compared to the octreotide-treated animals. This difference appears to be a non-statistically significant trend for hepatocytes at 1 week post-hepatectomy, but is gradually extended, resulting in a statistically significant difference at 2 months post-hepatectomy. This is by itself a most interesting finding, since at this point the macroscopic restoration of liver mass is almost complete. Therefore, this is indicative of a sustained long-term effect of octreotide on hepatocyte function and proliferation which remains to be further explored [24].
A particular question in the current research protocol was to test the hypothesis that estrogen receptor alpha suppression is a long term consequence of octreotide administration. This has indeed been demonstrated in short-term liver regeneration experimental trials but hasn’t so far been tested at a long-term setting [14, 25]. Under normal conditions ERα is dominantly expressed in the normal and regenerating rat liver at the level of the cytoplasm [6-8, 10], so it would be reasonable to assume that any direct effect of octreotide on the sex steroid axis would result in differential ERα expression. Being that ERα expression is also significantly diverse between hepatocytes and cholangiocytes (the biliary tree being in fact more rich in ERβ expression) the quantification of ERα immunohistochemical detection should therefore include both major cell populations examined separately and concomitantly.

In particular, in the control group, 168h post-hepatectomy, tissue samples examined verified a significant cholangiolar immunohistochemical reactivity for ERα which is also exhibiting a loco-regional variance, with the highest labeling index measured proximally to the surgical incision site. Reactive cholangiocytes form a series of ring or chain-like structures along the tissue, surrounded by regenerative hepatocyte inslets. These areas exhibit a considerable number of mitoses and a higher labeling index for ERα. Moreover, ERα detection in these areas is more abundant within the nuclei, thus implying the existence of activated ERα-estrogen complexes. In areas with more advanced liver regeneration, the creation of mature hepatocytes is characterized by low grade cytoplasmic ERα expression, which is more compatible to that observed in normal liver [6-8].

The comparison of ERα expression (labeling index) for both major cell populations in which quantification could be achieved at 168h post-hepatectomy revealed a statistically significant superiority for control animals compared to age-matched members of the intervention group. This finding verifies the experimental hypothesis and implies that octreotide continues to suppress ERα expression a week after partial hepatectomy, resulting in a concomitant delay in live regeneration rate, as has already been shown for the early steps of the process [20, 24].

With regard to long-term octreotide administration, the study of liver ERα expression at 2 months post-hepatectomy has revealed a so far unreported interaction among the two major hormonal cascades examined (i.e. the somatotroph and sex steroid axis). In particular, in normal control animals, the ERα labeling index increases for cholangiocytes in both intensity and distribution. With regard to loco-regional variation, the lowest expression is noted proximally to central vein branches. On the other hand, the large majority of both mature and regenerating hepatocytes are strongly stained for ERα. This immunohistochemical reaction is dominantly cytoplasmic, thus implying that the receptor is not activated and bound to estrogens at this point. Interestingly, certain groups of regenerating hepatocytes located proximally to cholangiocyte formations are not ERα reactive at this stage, a finding which might indicate a lower metabolic and proliferative activity for this particular cell group. On the other hand, hepatocytes located in zones 2 and 3 are consistently associated with a sustained, high intensity signal for ERα expression, a finding which is consistent with the normal adult liver expression pattern [6-8].

The comparative study of liver regenerative dynamics 2 months post-hepatectomy seems to further strengthen the validity of the study hypothesis. In particular, the immunohistochemical estimation of the ERα labeling index in age-matched control and octreotide-treated animals indicates a very significant suppression of its expression in the intervention arm of the study. This finding is of particular significance, since it signifies a sustained inhibitory effect of octreotide on liver ERα expression which persists even when liver regeneration seems to be macroscopically and histologically almost complete [20, 24]. This long-term effect is also supported by the observation that ERα expression is known to be normally increased over time in the regenerating liver [8, 13], while this is in fact reversed in the octreotide group between 168h and 1440h post-hepatectomy. Thus, there seems to be a competition between somatostatin and estrogen on the liver, which is not only limited to regeneration, but may also extend to normal liver as well [14, 25].

The potential cross talk between the somatotroph and steroid axis has relatively recently been examined in detail. In the case of the liver, the use of transgenic mice has verified that in the absence of the growth hormone receptor, liver regeneration is delayed and the typical molecular milestones of entry in the regenerative process (synchronized DNA replication, redistribution of cyclin A and D) are either totally missing or distorted [25]. Many of the molecules involved are expressed in a highly sexually dimorphic pattern in the normal liver and their concentration is directly associated with circulating estrogen and estrogen receptor levels [11]. Other mechanisms proposed to explain the crosstalk between somatotrophs and steroids have also been proposed [25].

In conclusion, our study has proven a long-term interaction between octreotide administration and the estrogen receptor type alpha liver expression. This may be attributed to any of the proposed mechanisms or even other alternative ones to be discovered in due course. Clarification of this phenomenon may prove crucial in the development of novel strategies for the prevention of hepatotoxicity and the acceleration of liver repair in a variety of clinical settings.

The authors declare that they have no conflicts of interest.

Bibliography


