Rat mesangial cells exhibit sex-specific profibrotic and proinflammatory phenotypes

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Abstract

Background. Chronic renal disease progresses more rapidly in males compared to females. This study investigated whether there were any inherent differences between male and female mesangial cells that could contribute to this phenomenon and whether these differences could be modulated by sex hormones.

Methods. Experiments were carried out on cultured mesangial cells derived from adult male and female Wistar rat kidneys. Fibronectin, TNFα and IL-1β levels were measured in control and macrophage-conditioned medium (MCM)-injured cells in the presence and absence of 17β estradiol or testosterone.

Results. Male mesangial cells expressed higher baseline fibronectin levels compared to female cells. Similarly, basal levels of the proinflammatory cytokines TNFα and IL-1β were higher in male cells. Fibronectin and IL-1β levels were enhanced proportionately between the sexes in response to MCM stimulation, whilst the increase in TNFα levels was greater in MCM-stimulated female cells. Treatment with 10−8 M estradiol down-regulated baseline fibronectin levels in female mesangial cells but had no effect on basal levels in male cells. Estradiol had no effect on MCM-stimulated fibronectin levels in female mesangial cells but further increased stimulated levels in male cells. Testosterone had no effect on basal fibronectin levels of either sex but further enhanced MCM-stimulated fibronectin levels in mesangial cells of both sexes. Sex hormone treatment had no effect on cytokine levels in male mesangial cells. However, in female cells estradiol decreased TNFα levels and increased IL-1β levels, while testosterone increased the levels of both cytokines.

Conclusion. These data would suggest that male mesangial cells inherently exhibit greater profibrotic and proinflammatory characteristics than female cells. The inherent gender phenotypes are further modulated by sex hormones. This sexual dimorphism in mesangial cells may play a contributory role in the faster rate of progression to end-stage renal disease in males.

Keywords: cytokines; 17β estradiol; fibronectin; mesangial cells; testosterone

Introduction

Male patients with diverse types of primary chronic kidney disease exhibit a more rapid rate of decline in renal function than do female patients [1]. This has been shown to be the case in primary inflammatory glomerular disease [2], diabetic nephropathy [3] and non-glomerular disease [4]. In prepubescent patients with hereditary renal disease, there is no sex-linked difference in age at time of death from renal disease [4] suggesting that sex hormones are the underlying determining factor in the development of renal failure rather than chromosomal gender per se. A meta-analysis using 68 studies and evaluating over 11 000 patients supported the hypothesis that men with non-diabetic chronic renal disease, irrespective of aetiology, show a more rapid decline in renal function than women [5]. However, the meta-analysis was unable to assess whether the presence of testosterone or the absence of oestrogen was the determining factor.

Many students in experimental animals have examined the direct effects of sex hormone treatment or effects of sex hormone withdrawal via ‘gonadectomy’ [6–8]. The majority of these studies support the hypothesis that oestrogens are generally protective and androgens are detrimental to the course of renal disease. However, in some models of renal disease, oestrogens have also been associated with an acceleration of the renal injury [9,10].

The development of glomerulosclerosis is one of the classic histological hallmarks of end-stage renal disease. The glomerulus has been shown to be an oestrogen-target tissue. Oestrogens acting via ERα receptors are thought to modulate the turnover of extracellular matrix protein in a manner that would be expected to protect against progressive scarring and the development of glomerulosclerosis [11,12]. Mesangial cells are thought to be primarily responsible for modulating the levels of extracellular matrix in the glomerulus. A number of in vitro studies have been conducted to examine the effects of sex hormones on mesangial cell pathophysiology [13–17]. Of note, the reduced expression of collagens I and IV in response to estradiol or selective oestrogen receptor modulators (SERM) has been demonstrated.

However, to our knowledge, no studies have been performed specifically to evaluate the role of chromosomal
gender per se on renal disease progression. Moreover, no studies have directly compared the profibrogenic potential of male and female kidney cells in vitro either basally or in response to injury. We have therefore examined the effects of gender on renal scarring using mesangial cells in culture. Specifically, we have evaluated whether there is an inherent sex-specific difference in their profibrotic potential. In addition, we have assessed how sex hormones might modulate any profibrotic responses both under basal conditions and under conditions in which mesangial cells have been stimulated by the macrophage-conditioned medium (MCM). We have previously demonstrated that exposure of mesangial cells to macrophage products (as might be expected to occur in vivo following renal injury) stimulates a profound profibrotic response [18] allowing the in vitro study of factors regulating the development of mesangial scarring in an environment devoid of confounding circulating and haemodynamic factors.

Materials and methods

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich Chemical Co. (Dorset, UK).

Cell culture

Glomerular mesangial cells were cultured from either the glomerular ex-
plants of age-matched adult male or female Wistar rat kidneys (Uni-
versity of Leicester colony) using standard techniques. The cells were
cultured in RPMI 1640 (Invitrogen, Paisley, UK) supplemented with
20% heat inactivated foetal calf serum (FCS), 100 U/ml penicillin,
100 µg/ml streptomycin, 5 µg/ml insulin and 2mM glutamine. Cultured
cells were characterized as mesangial by their typical stellate, fusiform
morphology, their positive staining for the Thy-1 antigen and their resis-
tance to the toxic effects of D-valine and PAN.

In all experiments, mesangial cells of passages 2 through 10 were cul-
tured in 24-well plates (Costar-Corning, Buckinghamshire, UK) or 25 cm²
flasks (Costar-Corning), allowed to grow to confluence, then rendered qui-
escent in the RPMI medium containing 0.5% FCS for 48 h prior to stimu-
lation. In preliminary experiments, we found that there was no significant
difference in fibronectin production between cells grown in phenol red
free and phenol red containing RPMI.

Unless otherwise stated, experiments were conducted on mesangial
cells derived from five different pairs of age- and sex-matched male
and female rats.

Preparation of the MCM

The MCM was prepared from LPS-stimulated, peritoneal macrophages
from either male or female rats as previously described [18].

Culture of mesangial cells in the presence of MCM

In order to examine the effects of mesangial cell injury conflu-
ent, quiescent male and female mesangial cells were exposed to a 50% solution of
MCM in the presence or absence of 10⁻⁸ M 17β estradiol or 10⁻⁸ M
progesterone. The cultures were maintained in this medium for 1 or 3 days.

The tissue culture supernatants were harvested and stored at −20°C
for subsequent analysis. In these experiments, female mesangial cells were
stressed with MCM derived from female rats and male mesangial cells
were stressed with MCM derived from male rats. Cross-over experi-
ments were also carried out where mesangial cells were stressed with
MCM generated from macrophages derived from animals of the opposite
gender.
male and female MCM to induce fibronectin, implying that the response was inherent in the mesangial cells themselves (Figure 2B).

**TNFα and IL-1β expression**

TNFα and IL-1β are pleiotropic cytokines involved in inflammation and scarring. Basal TNFα (Figure 3A) and IL-1β (Figure 3B) levels were higher in male than in female mesangial cells (165 ± 53 versus 82.5 ± 7.3 and 14.3 ± 5.3 versus 10 ± 1.4 pg/mg cell protein, respectively). MCM stimulation further elevated TNFα (Figure 3A) and IL-1β (Figure 3B) levels in male and female mesangial cells (412 ± 96 versus 281 ± 8.5 and 25.9 ± 5.6 versus 17.6 ± 0.8 pg/mg cell protein, for TNFα and IL-1β, respectively). However, the fold increase in TNFα levels was greater in female cells than in males (3.4 versus 2.5). There was no difference in the fold increase between the sexes in IL-1β levels (1.8 versus 1.7).

**Effect of sex hormones on fibronectin levels**

Control and MCM-stimulated male and female mesangial cells were treated with 10⁻⁸ M 17-β estradiol or testosterone. 10⁻⁸ M 17-β estradiol down-regulated basal fibronectin levels in female mesangial cells by 16 ± 2.4% (P < 0.001) but had no effect on MCM-stimulated female cells (Figure 4A). In male cells, estradiol had no effect on basal fibronectin levels but unexpectedly induced a small increase in MCM-stimulated cells (17.7 ± 2%, P < 0.02) (Figure 4A). Testosterone had no effect on basal fibronectin levels in cells of either sex (Figure 4B). However, fibronectin levels were further increased by 10⁻⁸ M testosterone in MCM-stimulated cells of both sexes (23 ± 6.9%, P < 0.03 and 32 ± 8.55%, P = 0.02 in male and female cells, respectively) (Figure 4B).

**Effect of sex hormones on cytokine levels**

Estradiol induced a small reduction in MCM-stimulated TNFα levels in female cells (10.7 ± 2.7%, P < 0.04). Testosterone, on the other hand, slightly up-regulated TNFα levels by 7 ± 0.6% (P < 0.005) in female MCM-stimulated mesangial cells (Figure 5A). IL-1β levels were elevated by both estradiol and testosterone in MCM-stimulated female
Fig. 3. TNFα (A) and IL-1β (B) levels in male versus female mesangial cells. TNFα and IL-1β levels were measured in control and MCM-stimulated male and female mesangial cells. Results are shown as means ± SEM (n = 3) expressed as pg cytokine per mg cell protein. Male mesangial cells expressed higher cytokine levels than female cells.

cells (22 ± 8.6%, P < 0.05 and 25 ± 8%, P < 0.04, respectively) (Figure 5B). Cytokine levels in MCM-stimulated male mesangial cells were not significantly affected by treatment with either hormone (Figure 5B).

Discussion

Historically, gender differences in renal disease susceptibility have been attributed to the protective effects of oestrogen, a hormone with known anti-inflammatory [19,20] anti-oxidant [21] and anti-fibrotic properties [22]. Investigations in mesangial cells have demonstrated that estradiol and its metabolites have the potential to be renoprotective inhibiting apoptosis and transforming growth factor (TGFβ) activity and expression [23], increasing the expression of extracellular matrix degrading metalloproteinases [24], reducing the synthesis of collagen types I and IV [15] and inhibiting cell proliferation [16]. In parallel experiments, addition of exogenous testosterone to mesangial cell cultures has shown a little effect on collagen synthesis or cell proliferation [16]. Few cell culture studies have assessed inherent differences in male and female cells in the context of their susceptibility to injury, and to our knowledge no one has compared the effects of gender per se on renal cell phenotypes. Such differences could, in part, account for the more rapid progression to end-stage renal disease seen in males with kidney disease.

The current study has demonstrated that male mesangial cells in culture exhibit a more fibrotic phenotype than female mesangial cells, secreting significantly more fibronectin, TNFα and IL-1β both constitutively and following injury, suggesting that male chromosomal gender bestows an inherent propensity to develop a fibrotic phenotype. Cultured lymphocytes from males have similarly been shown to produce more TNFα and IL-1β than those from female lymphocytes [25].

Intrinsic differences between male and female mesangial cells may be further modified in vivo by the prevailing hormonal environment. TNFα has been implicated in a number of chronic renal diseases [26] and is known to be involved in inducing renal fibrosis. Expression and function of this cytokine are also known to be modulated by sex hormones. For example, premenopausal women have lower lipopolysaccharide-induced TNFα levels than men or postmenopausal women [27]. Furthermore, levels have been shown to vary with the stage of menstrual cycle [28]. The results from the current study suggest that estradiol generally reduces and testosterone increases the profibrotic phenotype of cultured female mesangial cells. Sex hormone
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The 'sex' of the derived MCM influence of sex hormones, constitutively produce more mesangial cells themselves as male cells, outside of the influence of sex hormones, constitutively produce more fibronectin than females. The 'sex' of the derived MCM does not appear to play a role as in the cross-over MCM experiments, where male and female mesangial cells were exposed to MCM derived from animals of different sexes, there was no sex-specific difference in the ability of the MCM to induce fibronectin in mesangial cells. In further support of this, Huang et al. have shown that there is no significant difference between cytokine expression levels in peritoneal macrophages from male and female mice. Moreover, they found that even though oestrogen decreased, and progesterone increased cytokine expression levels by macrophages, there was no significant difference in the magnitude of the response to sex steroids between male and female peritoneal macrophages [32].

TGFβ is a fibrogenic growth factor known to be affected by estradiol [23], and estradiol has been shown to reverse the profibrotic effects of TGFβ in experimental animal models [19]. However, neither estradiol nor testosterone has previously been shown to affect steady state TGFβ mRNA levels in mesangial cells [16]. Moreover, we have previously shown that TGFβ only accounts for 20% of the fibronectin response of rat mesangial cells to MCM (as determined by neutralizing TGFβ antibodies) [18]. Increased fibronectin production by male mesangial cells undoubtedly involves a number of mechanisms, one of which may involve increased production of a number of cytokines and growth factors acting concert. However, what specific aspect of the male genotype that results in the production of increased cytokines is yet to be determined.

In our in vitro study, the hormonal environment to which cells were exposed in culture was regulated. However, we cannot rule out effects of hormonal preconditioning of the kidneys in vivo prior to mesangial cell preparation and the role this may play in the observed effects. Unfortunately, delineation of these effects is beyond the scope of the current study, although the observations provide a platform for further investigations. It is of note that others have shown that male kidneys do not seem to be influenced by lack of ERα suggesting that the 'kidney knows its sex' [33].

Traditionally, culture conditions for studying steroid hormone effects have involved the use of phenol red-free media. This is because a lipophilic impurity contained in the phenol red preparation has been described as a weak oestrogen agonist in oestrogen sensitive cells [34]. However, in preliminary experiments we found that there was no significant difference in fibronectin production between cells grown in phenol red free and phenol red containing RPMI. Therefore, all of our experiments were carried out in RPMI containing phenol red.

Other investigators in this field have used charcoal-stripped FCS in their culture media. However, charcoal stripping not only removes sex hormones but also other polypeptide growth factors whose activity may be required for normal healthy growth. For instance, charcoal-stripped sera have been shown to alter proliferation rates of MCF7 cells [35]. In preliminary studies, we found that mesangial cells are very sensitive to the culture conditions in which they are grown and we therefore used batch-tested FCS to ensure consistency.

To our knowledge, this is the first study to directly compare the differences in profibrotic responses between male and female mesangial cells. These data would suggest that male rat mesangial cells inherently exhibit greater

Figure 5. Effect of sex hormones on mesangial cell TNFα and IL-1β levels. TNFα (A) and IL-1β (B) levels were measured in male and female mesangial cell supernatants. Results are means ± SEM (n = 3) expressed as pg cytokine per mg cell protein. E = 17β estradiol, T = testosterone.

effects were less obvious in male mesangial cells although fibronectin levels in MCM-stimulated cells were increased by testosterone and to a lesser degree (and unexpectedly) by estradiol. Metcalfe et al. have previously demonstrated that testosterone up-regulates TNFα and profibrotic signalling in animals of both sexes in a model of obstructive renal injury when compared to control females or castrated males [29]. Other studies have shown that androgens up-regulate atherosclerotic genes in macrophages from males but not from females [30]. While oestrogen generally appears to convey protection, this is not universally the case for all experimental models of renal disease. In diabetic nephropathy and hyperlipidaemia, for example, female gender has been found to be a risk factor for renal disease [10,31]. Similarly, in vitro, the differential response between male and female cells may depend on the culture conditions and the type of stimulus applied. It is clear that the response of experimental animals or cells of different gender to sex hormones is also a multifactorial process that may vary according to the cell type and function, and disease aetiology.

As to the mechanism of the observed gender dimorphism, it is clear that the sex-specific phenotype is inherent in the mesangial cells themselves as male cells, outside of the influence of sex hormones, constitutively produce more fibronectin than females. The 'sex' of the derived MCM does not appear to play a role as in the cross-over MCM experiments, where male and female mesangial cells were exposed to MCM derived from animals of different sexes, there was no sex-specific difference in the ability of the MCM to induce fibronectin in mesangial cells. In further support of this, Huang et al. have shown that there is no significant difference between cytokine expression levels in peritoneal macrophages from male and female mice. Moreover, they found that even though oestrogen decreased, and progesterone increased cytokine expression levels by macrophages, there was no significant difference in the magnitude of the response to sex steroids between male and female peritoneal macrophages [32].

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profibrotic and proinflammatory characteristics than female rat mesangial cells. However, what specific aspect of the male genotype results in the profibrotic phenotype is yet to be determined. This sexual dimorphism in mesangial cells may play a contributory role in the faster rate of progression of glomerulosclerosis leading to end-stage renal disease in males. The inherent gender phenotypes are further influenced by circulating sex hormones that play an additional role in modulating the phenotype of the cells.

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