CCN-2 is up-regulated by and mediates effects of matrix bound advanced glycated end-products in human renal mesangial cells

Xiaoyu Wang · Susan V. McLennan · Stephen M. Twigg

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Abstract CCN-2, also known as connective tissue growth factor (CCN-2/CTGF) is a cysteine rich, extracellular matrix protein that acts as a pro-fibrotic cytokine in tissues in many diseases, including in diabetic nephropathy. We have published that soluble advanced glycation end products (AGEs), that are present in increased amounts in diabetes, induce CCN-2. However in vivo AGEs are known to be heavily tissue bound and whether matrix bound AGEs regulate CCN-2 has not been investigated. In this study we determined in human renal mesangial cells if CCN-2 is induced by matrix associated AGEs and if CCN-2 may then secondarily mediate effects of matrix AGEs on extracellular matrix expansion. Data generated show that CCN-2 mRNA and protein expression are induced by matrix bound AGEs, and in contrast, this was not the case for TGF- β 1 mRNA regulation. Using CCN-2 adenoviral anti-sense it was found that CCN-2 mediated the up-regulation of fibronectin and the tissue inhibitor of matrix metalloproteinase, TIMP-1, that was caused by matrix bound AGEs. In conclusion, CCN-2 is induced by non-enzymatically glycated matrix and it mediates downstream fibronectin and TIMP-1 increases, thus through this mechanism potentially contributing to ECM accumulation in the renal glomerulus in diabetes.

Keywords CCN-2 · Diabetic nephropathy · Advanced glycation · Matrix · CTGF

Introduction

Diabetic nephropathy is now the single commonest cause of end-stage renal disease in the developed world (Nathan et al. 2009), (Lehmann and Schleicher 2000). One of the proposed mechanisms is through the accumulation of advanced glycation end products (AGEs) (Cooper 1998). AGEs are a heterogeneous group of molecules formed from the non-enzymatic glycation on free amino groups of protein, lipids and nucleic acids, resulting in the rapid formation of a Schiff base, subsequently to a more stable Amadori product and leading to irreversible formation of end-products of the non-enzymatic glycation process (Bierhaus et al. 1998), (King and Wakasaki 1999).

AGEs are commonly elevated in serum (Chiarelli et al. 1999) and in many tissues in patients with diabetes (Beisswenger et al. 1995). However AGEs are not just longer term markers of elevated glucose as they have been shown to contribute to tissue pathology through two main mechanisms. Firstly, AGEs may covalently cross-link and biochemically modify protein structure and function, including cellular matrix, basement membranes, and vessel-wall components (Vlassara et al. 1986). Secondly, AGEs as ligands may bind to a variety of cell-surface AGE-binding receptors, and by this means, consequent induction of cytokines and growth factors by AGEs has been implicated in contributing to diabetic renal disease (Thornalley 1998), (Schmidt et al. 1994), (Vlassara 1992). In the studies of growth factors and cytokines, AGEs effects have usually been examined using AGEs added in solution (Bierhaus et al. 1998), (Twigg et al. 2002a), (King and Wakasaki 1999), and few studies have examined whether the matrix bound AGEs may affect cellular gene expression or function.

We were the first to report that CCN-2, also known as connective tissue growth factor (CTGF), at the mRNA and protein level is up-regulated by soluble advanced glycation

X. Wang · S. V. McLennan · S. M. Twigg (✉)
Sydney Medical School, The University of Sydney,
Sydney, NSW 2006, Australia
e-mail: stephen.twigg@sydney.edu.au

S. V. McLennan · S. M. Twigg
Department of Endocrinology, Royal Prince Alfred Hospital,
Sydney, Australia

end-products (AGEs) in vitro (Twigg et al. 2001), and that by this means CTGF/CCN-2 contributes to ECM induction (Twigg et al. 2002b). Subsequently, other groups confirmed those findings (Lee et al. 2004), (Lee et al. 2005), (Berlanga et al. 2005), (Chung et al. 2009). However, whether advanced glycation of matrix (matrix bound AGEs) induce CCN-2 has not been investigated.

Expansion of ECM is a pathological hallmark in most tissues affected by diabetes complications including diabetic nephropathy (Ziyadeh 1993), (Schleicher and Nerlich 1996). The ECM expansion has been proposed to be due to a combination of increased ECM production (Mason and Wahab 2003) and a reduction in ECM breakdown (Nakamura et al. 1994), (McLennan et al. 1994). CTGF/CCN-2 is a potent inducer of ECM in fibroblasts and it is elevated in the diabetic kidney in human diabetes (Adler et al. 2001), and in animal models (Murphy et al. 1999), (Riser et al. 2000). CTGF/CCN-2 is induced by high glucose conditions in human renal mesangial cells (Murphy et al. 1999), and consequentially as CCN-2 can regulate ECM induction, as well as proteases and their inhibitors (Wang et al. 2003), it may mediate ECM expansion and fibrosis in diabetic nephropathy through both induction of ECM formation and inhibition of ECM degradation (McLennan et al. 2004), (Twigg 2011).

Given this background, the aim of this study was to determine whether CCN-2 in human renal mesangial cells is regulated by AGEs which are bound to cell matrix, and by this means whether CCN-2 might mediate AGEs effects on ECM.

Material and methods

Mesangial cell isolation and culture

Human renal fetal mesangial cells were isolated and identified as previously described (McLennan et al. 1994), (McLennan et al. 2004) as approved by the University of Sydney Human Research Ethics Committee. Briefly, glomeruli were isolated by serial sieving from kidneys obtained from terminations of pregnancies in the second trimester. The mesangial cells were then cultured as outgrowths from the glomerular isolates in RPMI medium with 10% FCS in 5% CO₂ and air until confluence for passaging. All experiments were carried out in duplicate or triplicate in cells from P2-P4 passage, and were repeated using cells derived from different kidney sources.

Soluble AGEs synthesis and treatments

Soluble AGEs were prepared as previously described using a standardised approach with the carbohydrate D-ribose (McLennan et al. 2007), (Twigg et al. 2002a). In brief, bovine serum albumin (BSA) (Sigma, RIA grade, fraction V) as 10 mmol was co-incubated in sterile PBS with

500 mmol/L D-ribose for 10 d under aerobic conditions at 37°C (termed 'AGE-BSA'). Tubes with simultaneous incubations under the same conditions but without the addition of the D-ribose (termed 'control-BSA') were prepared as control for comparison with AGEs-BSA treatments. In parallel preparations, aminoguanidine at 100 mmol/L, as an inhibitor of formation of AGEs, was co-incubated with the 10 mmol BSA and the 500 mmol/L D-ribose (termed 'aminoguanidine-BSA'). All preparations were extensively dialyzed in PBS, using a low molecular weight cut-off membrane (6–8 kDa, Spectrapor 1, Spectrum Industries, Los Angeles, CA) to remove the free ribose and where relevant, the aminoguanidine. AGEs content was found to be five fold increased by fluorescence measurement of browning product compared with control BSA.

Soluble AGE-BSA, aminoguanidine-BSA, or control BSA, each at a standard dose of 100 µg/ml BSA (Twigg et al. 2001), (Twigg et al. 2002a), were applied for 72 h onto the mesangial cells. After cell treatments, total RNA was isolated and reverse transcribed, and CCN-2 mRNA and fibronectin mRNA and TGFβ1 were then measured by quantitative real-time RT-PCR.

Glycation of mesangium matrix

Advanced glycation of mesangial matrix was prepared using a standardised method as we have previously reported (McLennan et al. 2002). In brief, the cultured mesangial cells were trypsinized, and passage to six-well plates (Iwaki, Crown Scientific), and grown in RPMI medium containing 11 mmol/l D-glucose and 10% FCS. At confluence, medium was decanted and the cells were washed with PBS and removed from the matrix by incubation in 0.25 M NH₄OH at 37°C for 30 min. Then after washing extensively with distilled water, the matrix was fixed with 70% ethanol and sterilized by exposure to ultraviolet light for 10 min. The matrix was then glycated by incubation in PBS (pH 7.4) containing 500 mmol/l D-ribose at 37°C for 10 days as previously described (Krishnamurti et al. 1997). Matrix incubated in PBS alone was used as control. Aminoguanidine, an inhibitor of AGEs formation, was added to act as another negative control for AGEs formation (McLennan et al. 2002). In some experiments, 100 mmol/L aminoguanidine (AG) (Sigma, Sydney, Australia) was added to the 500 mmol/L D-ribose solution to inhibit the formation of AGEs. By fluorescence, glycation was increased three fold in the glycated matrix compared with control matrix. Isolated human mesangial cells were later subsequently grown on the prepared matrix for up to 96 h.

Adenoviral anti-sense

Cells cultured on the respective matrix for 48 h were each treated with adenoviral CCN-2/CTGF antisense or adenoviral

vector (10^{10} infectious particles/mL each as previously reported (Burns et al. 2006)) for a further 48 h in serum-free fresh RPMI media containing 0.1% BSA. Effects of these matrices on fibrotic cytokines CCN-2, fibronectin, TGF- β 1 and tissue inhibitor of matrix metalloproteinases (TIMP-1) were examined. In some experiments rhTGF β 1 purchased from R&D Systems, Inc. (Minneapolis, MN) at 0.1 and 1.0 ng/mL was also added, as indicated.

Gene expression measurement by real-time RT-PCR

Mesangial cells were grown to confluence on glycosylated and nonglycosylated matrices as described, or on regular plastic wells and then total RNA was isolated and RT-qPCR performed similar to previous described methods (Twigg et al. 2002a), (Twigg et al. 2001). In brief, cultured cells in 6-well plates were then washed twice with PBS, total RNA was isolated from cells in triplicate using the RNeasy Mini kit (Qiagen, Melbourne, Australia), and the RNA concentration was determined with the use of the SYBR green RNA assay (Molecular Probes, Eugene, OR). Each reaction mixture contained 1 μ g of total RNA and was reverse-transcribed into cDNA using Random Hexamer Primer (Invitrogen, CA, USA) and SuperScriptTM III Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The resulting cDNA was analyzed by quantitative real-time PCR using ABI 7700, using (Applied Biosystem). The CCN-2/CTGF, fibronectin genes were amplified by sequence-specific primers with Taqman probe and the TIMP-1 and TGF- β 1 gene were amplified by using SYBR green. For CCN-2/CTGF: forward, 5'-GAGGAAAACATTAAGAAGGGCAAA-3'; reverse, 5'-CGGCACAGGTCTTGATGA-3'; probe 5'-6FAM-TTTGAGCTTTCTGGCTGCACAGTGT-TAMRA-3'; For fibronectin (FN): forward, 5'-TCCTTGCTGGTATCATGGCAG-3'; reverse, 5'-AGACCAGGCTTCTCATACTTGA-3'; probe, 5'-6FAM-CCACGTGCCAGGATTACCGGCTACAT-TAMRA-3'; For TIMP-1: forward, 5'-TACTTCCACAGGTCCACAACC-3'; reverse, 5'-GGCTATCTGGGACCGCAGGACTGCCA-3'; For TGF β 1: forward, 5'-AGGTCACCCGCGTGCTAAT-3'; reverse, 5'-TCAACCACTGCCGCACAACT-3'. In each case, the gene of interest expression level was normalized to 18 s ribosomal RNA (purchased from Applied Biosystem for Taqman probe and primers) and related to the relevant control, as previously described (Twigg et al. 2001) and as indicated in the text.

Western immunoblot analysis

Cells in 6-well plates treated in each case in triplicate were washed with cold PBS, then 150 μ l of cold RIPA lysis buffer with a complete protease inhibitor cocktail at concentration recommended by the manufacturer (Roche

Diagnostics, NSW, Australia) was added to each well. Plates were left on ice for 20 min, and lysates were collected after centrifugation at 10,000 g for 10 min at 4°C. Total protein concentration was measured using the DC Protein Assay (Bio-Rad Laboratories, Richmond, CA), to normalise sample loading for SDS-PAGE.

Cell lysate samples were loaded at 50 μ g of total protein per lane and separated on a 12% SDS-PAGE gel under reducing conditions. Proteins were electrotransferred onto nitrocellulose, and membranes were blocked with 5% non-fat dry milk/TBS with 0.1% (Vol/Vol) Tween 20 for 1 h, followed by incubation with in-house anti-CTGF IgG (1:1000, termed 194) (Thomson et al. 2008), (Brooks et al. 2008). After further washes, incubation of membranes with the appropriate horseradish peroxidase (HRP)-labeled secondary antibody (1:10,000, Vector Laboratories) for 1 h at 21°C occurred. Immunoreactive protein bands were then detected using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA). In addition to loading the same calculated total protein in each lane of the gel, a non-specific high molecular weight band found to be present in the cell lysates was used as a protein loading control.

Statistical analysis

Results are expressed as the mean value $\pm$ SD. All numerical data were derived from three or more independent experiments. Differences between groups were assessed by one way ANOVA with bonferroni's correction for multiple comparisons. $P < 0.05$ was considered statistically significant.

Results

Effect of matrix bound AGEs and soluble AGEs on CCN-2 in mesangial cells

To examine effects of soluble AGEs treatment, gene expression of CCN-2, fibronectin and TGF β 1 were measured by RT-qPCR after exposure of human renal mesangial cells in monolayer to 100 μ g/mL of AGEs-BSA, control-BSA or aminoguanidine-BSA for 72 h. The gene expression of each was found to be significantly induced by soluble AGEs treatment (Fig. 1a, b and c). In parallel experiments, to assess whether matrix glycation affects fibrotic gene markers, human mesangial cells were grown on the glycosylated matrix, the non-glycosylated control matrix or the aminoguanidine matrix and gene expression of CCN-2, fibronectin, and TGF β 1 were measured. CCN-2 mRNA was found to be induced by glycosylated matrix and as expected, this induction did not occur with matrix pretreated by aminoguanidine (Fig. 1d). Fibronectin expression

Soluble AGEs

Matrix-bound AGEs

Fig. 1 Soluble and matrix bound AGEs effects on CCN-2, fibronectin and TGF-β1 gene expression. Mesangial cells in monolayer were treated by soluble phase AGEs as glycated BSA, or control BSA (each at 100 μg/ml), for 72 h. Gene expression of CCN-2, fibronectin and TGF-β1 are shown in **a–c**, as mean ± SD, *, $P < 0.05$ vs control matrix. The effects of AGEs bound to matrix on the same genes was then examined. Mesangial cells (P2–P4) were grown in the 6 well-

plates containing control, glycated matrix or aminoguanidine matrix and cells were harvested after 72 h. The mRNA expression of CCN-2, fibronectin (FN) and TGF-β1 (**d–e**) in cells grown on the different matrices were measured by qRT-PCR. Data is shown as mean ± SD, *, $P < 0.05$ vs the control matrix for the respective mRNA species. Experiments were independently repeated 3 times

was mildly and non-significantly induced (Fig. 1e). In contrast to effects observed for CCN-2 grown on glycated matrix (Fig. 1d) and for effects of soluble AGEs on TGFβ1 (Fig. 1c), TGFβ1 mRNA expression was reproducibly inhibited when cells were grown on glycated matrix, compared with control matrix or the amino guanidine pre-treated matrix (Fig. 1f).

CCN-2 mediated induction of TIMP-1 by glycated matrix

As CCN-2 was found to be induced by glycated matrix, to then test if CCN-2 induction may mediate effects of glycated AGEs matrix on pro-fibrotic gene markers, adenoviral CCN-2 antisense or its control of vector alone were applied to the mesangial cells which has been grown on the control, glycated and pre-treated aminoguanidine cell matrix. CCN-2 mRNA and cell lysate protein expressions induced by matrix glycation were blocked by CCN-2

antisense (Fig. 2a and b). CCN-2 antisense also prevented the TIMP-1 induction by glycated matrix, compared with the CCN-2 vector control alone (Fig. 2c). Fibronectin mRNA showed the same trend as for TIMP1 (Fig. 2d). In contrast, no effect of CCN-2 antisense on TGFβ1 mRNA expression in control, glycated or glycated plus amino guanidine matrix was observed (Fig. 2e) and TGFβ1 mRNA expression was found to be reduced in aminoguanidine pre-treated matrix.

TGF-β1 enhanced fibrotic cytokines expression in glycated matrix

In some cell systems including renal cells CCN-2 may up-regulate TGF-β1 signaling pathways and bioactivity (Twigg 2011). In a final series of experiments we applied rhTGF-β1 at low dose onto cells grown on the different matrices and examined downstream effects on fibronectin

Fig. 2 CCN-2 effects on pro-fibrosis in the presence of AGEs bound matrix. Cells grown on the control matrix, glycated matrix or aminoguanidine matrix for 48 hr were treated with adenoviral CCN-2 antisense or adenoviral vector alone (10^{10} infectious particles/mL) for 48 hr. (a), CCN-2 mRNA was measured by real-time RT-PCR. Data is shown as mean $\pm$ SD. *, *P*<0.05, for glycated matrix vs control matrix each with adenoviral vector alone; # *P*<0.05 for glycated matrix treated with adenoviral CCN-2 antisense vs glycated matrix treated with adenoviral vector alone. (b), The intact CCN-2 protein level was determined by

Western immunoblot of cell lysates. The intact CCN-2 at ~37 kDa is shown and the lower panel is a high molecular mass (115 kDa) non-specific control band used as a protein loading control. (c–e), TIMP-1, fibronectin and TGF- β 1 mRNA were measured by real-time RT-PCR. Data is mean $\pm$ SD. **, *P*<0.01, for glycated matrix vs control matrix each with vector alone; #, *P*<0.05, for CCN-2 antisense treated cells on glycated matrix vs vector alone treated cells on glycated matrix; !, *P*<0.05 for vector alone treated cells on aminoguanidine matrix vs vector alone treated cells on control matrix

Fig. 3 TGF- β 1 treatment effects on pro-fibrotic cytokine expression in cells grown on glycated matrix. Mesangial cells were grown in 6 well-plates containing control matrix, glycated matrix or aminoguanidine matrix for 48 h and then rhTGF- β 1 (0.1 or 1 ng/mL) was

applied for 48 h. The mRNA levels of fibronectin in (a) and TIMP-1 in (b) were examined by real-time PCR. Data is shown as mean $\pm$ SD. **P*<0.05, vs no rhTGF- β 1 addition on glycated matrix

and TIMP-1 gene expression. The rhTGF- β 1 treatment at low dosage (0.1 ng/ml) increased the fibronectin and TIMP-1 mRNA in glycated matrix at, indicating that glycated matrix may augment rhTGF- β 1 effects. Interestingly, a higher dose of rhTGF- β 1 had no effect to induce fibronectin or TIMP-1 mRNA species when the mesangial cells were cultured on control matrix, glycated matrix or aminoguanidine matrix (Fig. 3a–b).

Discussion

One main mechanism whereby elevated glucose may exert its effect on CCN-2 is through formation of non-enzymatic glycated proteins, with irreversible formation of AGEs (Bierhaus et al. 1998). AGEs may have pathological effects on tissues through activation of cell surface AGEs receptors and then post-receptor signalling or by direct effects of glycation on and thus impairment of, function of proteins, lipids, RNA and DNA (King and Wakasaki 1999). As shown schematically in Fig. 4, the current work demonstrates the novel findings that the growth factor CCN-2 is induced by matrix bound AGEs, and that CCN-2 then contributes to induction of gene expression changes to cause ECM formation and inhibition of ECM degradation.

Fig. 4 Schematic figure showing a key role for CCN-2 in mediating matrix accumulation by AGEs in renal mesangial cells. Diabetes mellitus induces CCN-2 through AGEs and the CCN-2 then causes both induction of new ECM synthesis as well as inhibition of ECM degradation, to cause ECM expansion in renal tissue. The new data generated by the current work is shown in bold arrows, where matrix bound AGEs induced CCN-2 and in turn CCN-2 induces the ECM marker FN and the broad spectrum inhibitor of matrix MMPs, TIMP-1. In contrast, while TGF- β 1 induces CCN-2 in renal mesangial cells, it does not appear to be an important factor in effects of AGEs on CCN-2

As the main cell type thought to cause diabetic glomerulosclerosis is the renal mesangial cell (Murphy et al. 1999), it is relevant that the main findings reported here are in primary cultures of human renal mesangial cells.

This report focuses on induction of CCN-2 by AGEs. Diabetes may induce CCN-2 through multiple mechanisms that include haemodynamic as well as metabolic mediators (Twigg 2011). It is recognised that mechanisms other than AGEs may induce CCN-2 in diabetes and these include the renin-angiotensin-aldosterone system (Liu et al. 2007), (Gauer et al. 2007), endothelin-1 (Shi-Wen et al. 2007), free fatty acids (Wang et al. 2009) and effects of glucose through non-AGEs dependent processes including reactive oxygen species (Park et al. 2001) and protein kinase C pathways (Murphy et al. 1999). To what degree the AGEs pathways may be more or less relevant compared with these other cell mechanisms was not explored by the current in vitro studies. However we have previously reported that systemic administration of the inhibitor of AGEs formation, aminoguanidine, largely prevented CCN-2 increases in the diabetic kidney in a rodent model of diabetes (Twigg et al. 2002a) and that in vivo data does implicate AGEs pathways as being a main mechanism by which diabetes causes renal CCN-2 increases.

AGEs in diabetes form both intracellularly and in the extracellular environment, and they are also thought to potentially be secreted from cells, creating an autocrine or paracrine AGEs link (Bierhaus et al. 1998). Most studies of AGEs have examined soluble extracellular administration and secondary cellular effects. In studies of soluble AGEs we reported that one cell surface receptor for AGEs, known as RAGE, mediated induction of CCN-2 to a large degree, albeit in primary cultures of human dermal fibroblasts (Twigg et al. 2001). The cell mechanism(s) by which matrix bound AGEs up-regulate cell expression of CCN-2 as shown in this current work, remains to be determined. While AGEs that are matrix bound may activate AGEs cell surface receptors, it is possible that the AGEs modified matrix secondarily causes CCN-2 induction rather than through AGEs cell receptors, for example through changing ECM protein function of for example, integrins sub-types or through focal adhesion kinase (Krishnamurti et al. 1997), and further studies will be required to address which mechanism(s) apply.

TGF- β 1 is a potent inducer of CCN-2 including in renal mesangial cells. However mechanisms other than TGF- β 1 may induce CCN-2. We and others have previously reported that TGF- β 1 does not mediate soluble AGEs effects to induce CCN-2 (Twigg et al. 2001). In the current work, we found that TGF- β 1 gene expression was actually down-regulated by matrix-bound AGEs whereas it was induced by soluble AGEs. These findings for TGF- β 1 reflect those that we have observed earlier (McLennan et al. 2002). This reproducible differential regulation of TGF- β 1

by the different phases of glycated matrix is striking and it indicates that cell mechanisms of effect for the two forms of AGEs may differ. As CCN-2 is known to augment TGF- β pathway signalling in renal mesangial cells through multiple potential mechanisms (Wahab et al. 2005) we tested if a low dose of rhTGF- β 1 on FN and TIMP-1 mRNA may be augmented by matrix-bound AGEs. The data indicate that while these solid phase formed AGEs do not induce TGF- β 1 gene expression, their presence does potentiate rhTGF- β 1 protein effects when applied to the cells. It can be speculated that the CCN-2 induced by matrix-bound AGEs may augment the effects of rhTGF- β 1, an hypothesis which would require formal studies blocking CCN-2 in the presence of added rhTGF- β 1.

CCN-2 induces ECM formation and inhibits ECM degradation. Our earlier work studying effects of CCN-2 has shown that it induces the matrix metallo-proteinase inhibitor, TIMP-1 and consequent inhibition of matrix degradation (McLennan et al. 2004), and others have reported in other cell systems that it causes induction of another main inhibitor of protease systems, tissue plasminogen activator inhibitor type 1 (PAI-1) (Zhang et al. 2007). The current work using CCN-2 antisense suggests that matrix bound AGEs effects on ECM formation as well as ECM degradation through TIMP-1 induction, are CCN-2 dependent. To further examine this hypothesis complex studies including those of matrix degradative activity (McLennan et al. 2004) would be indicated.

In summary, the current work addresses a further mechanism whereby diabetes as glycated matrix may work through CCN-2 to cause renal pathology. CCN-2 levels in the diabetic baboon (Thomson et al. 2008) and human kidney (Adler et al. 2002) appear to predict progressive albuminuria and blocking renal CCN-2 bioactivity prevented pathological albuminuria in a rodent model of diabetes (Guha et al. 2007). Phase I clinical trials inhibiting CCN-2 in human diabetic nephropathy are demonstrating some promise (Adler et al. 2010), (Twigg 2011). Whether targeting CCN-2 or advanced glycation in humans will prevent diabetic renal disease will ultimately be resolved by later phase human clinical studies and the tools to obtain such answers are increasingly being realised (Candido et al. 2003), (Adler et al. 2010).

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