Distinct functions for Ras GTPases in the control of proliferation and apoptosis in mouse and human mesangial cells

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In previous work, we have demonstrated that Ras GTPases regulate proliferation in a range of human renal cells. The present work compares human and mouse mesangial cell (HMC and MMC) responses to specific knockdown of Ras genes with antisense oligonucleotides (AS-oligos), and examines the role of the p21 (cip1) and p27 (kip1) cyclin-dependent kinase inhibitors in these responses in mouse cells. HMC and MMC were lipofectin transfected with ras-targeted AS-oligo at 200–400 nM for 18 h followed by growth of cells in 20% serum for 18–72 h. Cell proliferation was assessed with an MTS assay and bromodeoxyuridine (BrdU) uptake. Apoptosis was quantified using nuclear stain with Hoechst 33342 dye. In MMC, Ha-ras AS-oligo caused an increase in apoptosis from $<2\%$ to 10–15% of cells after 18 h in serum ($P<0.01$). Control, Ki-ras and N-ras AS-oligos had minimal effects on apoptosis. BrdU uptake studies showed that BrdU + ve MMC were increased by 20–40% ($P<0.05$) after Ha-ras AS-oligo at 24 h; other ras AS-oligos were inactive. HMC number was reduced by 40–80% ($P<0.01$) at 48–72 h by both Ha-ras and Ki-ras AS-oligos. These actions were associated with reductions in BrdU + ve cells. In HMC, the ras AS-oligos did not induce apoptosis. p21(−,−) MMC showed exaggerated apoptotic responses to Ha-Ras AS-oligo. In mouse cells, Ha-Ras expression appears necessary to prevent apoptotic cell death; Ras expression does not appear necessary for cells to progress through the cell cycle. In human cells, Ras does not appear necessary to prevent apoptosis but Ha-Ras and Ki-Ras appear to be required for cell cycle progression.

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Renal disease is often characterized by dysregulation of proliferation and apoptosis.[1] Abnormal mesangial cell proliferation is characteristic of several forms of human glomerulonephritis and is associated with excess glomerular cellularity, and with the damaging process of glomerular sclerosis.[2] An understanding of the cellular and molecular basis for this response and the cell signaling pathways involved is likely to lead to new targets for future therapies. There has been considerable progress in dissecting the likely extracellular factors that drive the proliferative response, and the mitogenic cytokine platelet-derived growth factor appears to have an important role.[3–5] There is less complete understanding of the intracellular signaling involved.

Ras monomeric GTPases play a critical role in the control of cellular proliferation, differentiation and apoptosis, and are potential targets in renal therapies.[6–9] There are three different isoforms of Ras: Harvey (Ha-Ras), Kirsten (Ki-Ras) and Neural (N-Ras). However, the distinct functions of these species are poorly understood. The upstream signals that control Ras activation include platelet-derived growth factor, fibroblast growth factor, epithelial growth factor and a range of other mitogens that have been implicated in renal disease. We have demonstrated in earlier work that both Ki-Ras and Ha-Ras are required for cell cycle progression in human renal fibroblasts.[7] The present work presents a study of the role of the Ras genes in mesangial cells.

In classical cell biological models, such as NIH 3T3 cells, there is abundant evidence for the role of the Ras family of monomeric GTPases in the control of cell proliferation.[6] The three ras genes are all potent transforming agents when mutated to be locked in the active GTP-bound form. Recent studies have shown that renal cell proliferation is also governed at the level of the cell cycle by regulatory proteins. Specifically, cyclin-dependent kinase (CDK) inhibitors, including p21 and p27, limit renal cell proliferation by binding to and inhibiting cyclin–CDK complexes.[10] However, the regulation of the CDK inhibitors p21 and p27 is not well understood in renal cells. There is recent evidence that the transcription of p21 and the expression level of functional p27 can be regulated by Ras in non-renal cells.[11–13] However,

in renal mesangial cells the relationships between the Ras isoforms and p21 and p27 have not been defined.

The goal of the current work was to determine the roles of the Ras GTPases in the control of proliferation and apoptosis in renal mesangial cells using ras antisense oligonucleotides (AS-oligos), and to examine the possible interactions between Ras and the CDK inhibitors p21 and p27. The results obtained show that the roles of Ras genes are quite distinct when mouse and human cells are compared. In human cells, the Ki-Ras and Ha-Ras GTPases are required for stimulated proliferation; in mouse cells, Ha-Ras is an antiapoptotic survival signal but Ras is not needed for proliferative signaling.

RESULTS

Cell number is altered by suppression of specific Ras isoforms

Figure 1 shows results determining the activity and specificity of the ras AS-oligos using linear-range reverse transcriptase-polymerase chain reaction (RT-PCR). Figure 1a displays RT-PCR products using Ki-Ras(4B) primers on mRNA from human mesangial cells (HMC) transfected with each of the four AS-oligos. Incubation with the Ki-Ras AS-oligo at 200 nM for 16 h abolished the Ki-Ras(4B) RT-PCR product. The 18S ribosomal bands were used to ensure equal loading in each sample. The Ha-Ras and N-Ras AS-oligos and control AS-oligo did not alter the levels of Ki-ras mRNA. Similarly, Figure 1b shows that the Ha-Ras AS-oligo specifically reduced Ha-Ras mRNA, which was not altered by the other AS-oligos. Figure 1c similarly shows that the N-Ras AS-oligo is specific in depletion of mRNA for its target isoform. These results demonstrate specific actions of the three ras antisense oligos on the levels of cellular mRNA for each isoform. Ras protein half-life has been determined by pulse chase to be 18–24 h, and the specific knockdown of Ras protein 48 h after transfection was found to be >80% (data not shown) as reported previously for renal fibroblasts.[7]

In mouse mesangial cells (MMC), the control AS-oligo was found to have no effect on the cell number curves when compared with lipofectin-alone mock transfection conditions. Figure 2a shows the cell number curves over 72 h for wild-type MMC after transfection with each of the three Ras AS-oligo compared with control AS-oligo transfection. As expected, there was an increase in the cell count with time under control conditions. This was not altered by transfection with N-ras or Ki-ras oligo. In contrast, there was a small but significant suppression of the growth curve at 24 h after Ha-Ras AS-oligo transfection but cell numbers were unaffected at 48 and 72 h.

The growth curves for p21(−,−) MMC are shown in Figure 2b. Cell number was not significantly altered in cells transfected with Ki-Ras and N-Ras AS-oligo compared with control AS-oligo. In contrast, there was significant growth suppression after transfection with the AS-oligo targeting Ha-Ras at 24, 48 and 72 h ($P<0.01$ versus control).

Figure 2c and d shows the results obtained in p27(−/−) and double knockout p21(−/−)p27(−/−) MMC, respectively. Transfecting these cells with each of the ras isoform AS-oligos did not significantly alter cell number at 24–72 h.

The cell number curves for HMC are shown in Figure 3. In human cells, the control AS-oligo caused a nonspecific reduction in cell numbers at 48–72 h. A nonspecific action

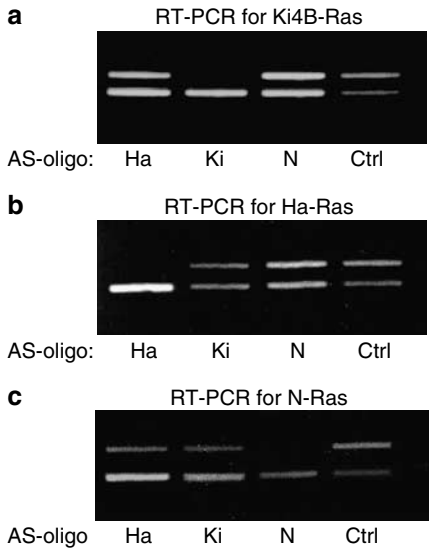

Figure 1 | Demonstration of specific actions of the ras oligos. In each PCR panel, the upper band represents ras and the lower band represent 18S ribosomal RNA control. (**a**) RT-PCR for Ki-ras after transfection with each of the three ras oligos (Ha-ras, Ki-ras, N-ras) and control oligo (ctrl). Similar results (**b**) for Ha-ras RT-PCR and (**c**) for N-ras are shown.

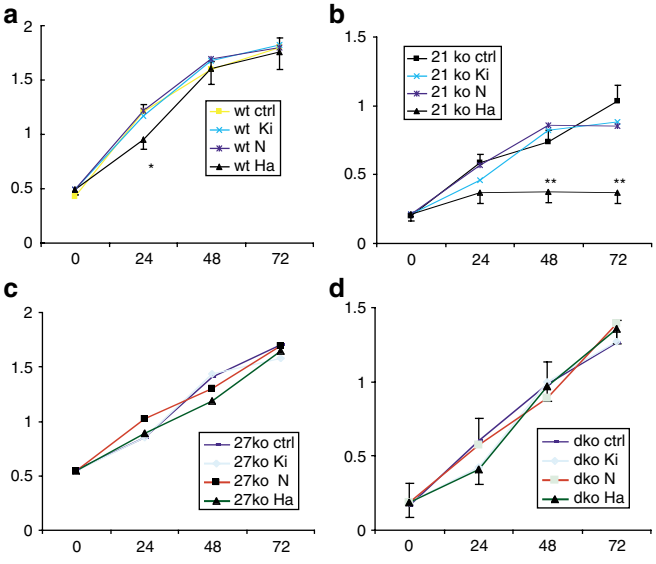

Figure 2 | Cell number as a function of time as monitored by the MTS assay for MMC, and the actions of Ras knockdown by isoform-specific oligos. The ordinates show cell number expressed as arbitrary units. (**a**) Data from wild-type MMC, (**b**) data from p21(−,−) MMC, (**c**) results for p27(−,−) MMC and (**d**) data for the double knockout p21(−,−) p27(−,−) MMC. Error bars are s.e.m. Each figure represents data from three independent experiments, each performed in quadruplicate ($n=12$). *$P<0.05$ versus control oligo data; **$P<0.01$ versus control oligo data.