impact cannot be discerned by the first-difference method.

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Nicholls replies — Godden *et al.* state that I proxied non-climate factors "by a linear trend in yields". I did not and my conclusions were not dependent on the functional form of any relationship between non-climate factors and yield. They suggest that forcing the intercept to zero and fitting with ordinary least-squares biases my estimates. I repeated the analysis without forcing a zero intercept, with no significant effect on the results. The climate–yield change relationship is so strong (see Fig. 2 of Ref. 1) that other forms of fitting produce a result very similar to ordinary least-squares. This relationship dominates the effects of other variables and provides a way to estimate the effect of climate on yield change. Godden *et al.* believe that detrending with first differences is inappropriate. As I noted, repeating the analysis on residuals from fitted non-linear trends produces very similar results.

Gifford *et al.* suggest that the temperature–yield relationship simply reflects the effect of rainfall on yield. Although rainfall was related to yield and temperature, the temperature–yield relationship was stronger. Even if the temperature–yield relationship partly reflects the influence of rainfall, there is a direct temperature effect as well. They believe the correlation between minimum temperatures and yield is unlikely to reflect the effect of frost damage. Yet there is a substantial literature showing concern about the effects of frost on Australian wheat yield.

Gifford *et al.* propose the use of multiple regression of absolute yield on climate variables. Many other factors affect yields. These would confound the use of such a regression of absolute yield on climate variables. Detrending, either through first-differencing or calculating residuals from fitted non-linear trends (I did both), is necessary to avoid this confounding. They suggest that regression on absolute yields would avoid underestimating the impact of the atmospheric CO_2. I did not need to, and did not, estimate the CO_2 impact on yield, but did check that the increasing CO_2 had not confounded my analysis. It had not.

Godden *et al.* restate my comment that farmers varying their inputs in response to climate might lead to an overestimate of the effect of climate. For instance, in a year with poor yield the farmer might decide not to harvest. Next year, with good climate conditions and good yield, the farmer harvests. The observed increase in yield, from one year to the next, could be attributed to increased harvest effort. However, the ultimate cause of the increased yield is the better climate, even if this is mediated partly by farmers' responses.

The comments of Godden *et al.* and Gifford *et al.* do not negate the conclusion that climate trends seem to have led to increased Australian wheat yield. Their comments indicate the difficulties in calculating the precision of the estimated yield increase owing to climate trends. Because of these difficulties, I did not attempt to calculate the precision of the estimate.

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Injected cytochrome *c* induces apoptosis

Mitochondria, the cell's energy-producing organelles, are thought to play a central role in mediating apoptosis, or programmed cell death. Mitochondrial morphology remains intact during the process, the apoptosis-blocking protein Bcl-2 is localized in the outer mitochondrial membrane[1], several critical steps in the process require ATP[2], and mitochondrial 'megachannels' or 'permeability transitions' open during apoptosis. It has been suggested that mitochondrial constituents leak out through these permeability transition channels, and activate the apoptotic machinery in the cytosol[3]. Here we show that the injection into cells of a key mitochondrial protein, cytochrome *c*, activates apoptosis.

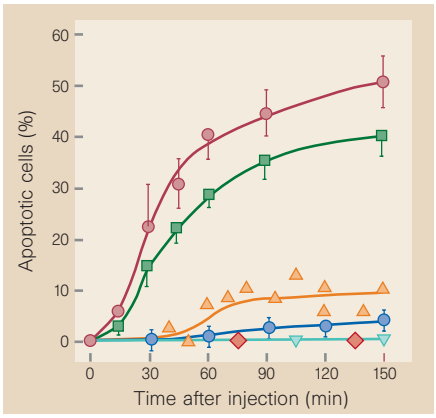

Figure 1 Effect of cytochrome *c* microinjection in NRK cells. Wild-type cells were injected with 20 μM (purple) or 10 μM (green) cytochrome *c*, with vehicle alone (turquoise) or with 20 μM biotinylated cytochrome *c* (red). Wild-type cells were pre-incubated with Z-VAD-fmk (30 min) and injected with 20 μM cytochrome *c* (blue). Cells overexpressing Bcl-2 were injected with 20 μM cytochrome *c* (orange). Each point represents the mean of at least four experiments involving injection of at least 50–100 cells.

Recently, three apoptotic protease activating factors (Apaf-1–3) were purified using a cell-free system based on cytosol from normally growing cells. One of these, Apaf-2, was identified as cytochrome *c*. This suggests that mitochondria may release cytochrome *c* during apoptosis[4]. Conversely, Bcl-2 has an inhibitory role in this translocation of cytochrome *c*, and prevents the activation of the cytosolic caspases and apoptosis[5,6].

We used a microinjection technique to load different cell types with cytochrome *c*. Types tested included adrenocortical Y-1 tumour cells, normal rat kidney (NRK) epithelial cells, mouse embryonic Swiss 3T3 fibroblasts and rat promyelocytic IPC-81 leukaemia cells. In all these systems, cytochrome *c* was able to kill cells by apoptosis in a dose-dependent manner (Figs 1, 2b), death occurring within 30–45 minutes (Fig. 1). Ninety minutes after injection — when the estimated cytochrome *c* intracellular concentration[7] was 20 micromolar — apoptotic morphology was shown by 46.7±4.7% of NRK, 52.6±7.2% of Y-1, 43.5±15.5% of Swiss 3T3 fibroblast and 28.3±4.3% of IPC-81 cells. We verified apoptosis by both co-staining with FITC-conjugated Annexin V and propidium iodide, and electron microscopy (not shown).

This apoptotic effect was found to be due to active cytochrome *c*, as injection of vehicle or of inactive, biotinylated cytochrome *c*[8] proved ineffective (Fig. 1).

Previously, cytochrome *c* has been shown to trigger caspase activation in cell-free extracts[4]. This conjecture was confirmed when we used cells that had been preincubated (for 30 minutes) with the caspase inhibitor Z-VAD-fmk; at 90 minutes after injection, less than 9.0% were apoptotic (Figs 1, 2c, d).

Cells that overexpressed Bcl-2 were also injected with cytochrome *c*. We found they were partially resistant to induction of killing: at 90 minutes, the level of apoptosis was reduced to approximately 30% of that seen in wild-type cells.

To ensure that we were correctly injecting cells preincubated with Z-VAD-fmk or overexpressing Bcl-2, we co-injected cytochrome *c* with TRITC and analysed the cells by fluorescence microscopy. The results showed that in both cases cells were properly injected. Thus, the protective effect depended on Z-VAD-fmk treatment

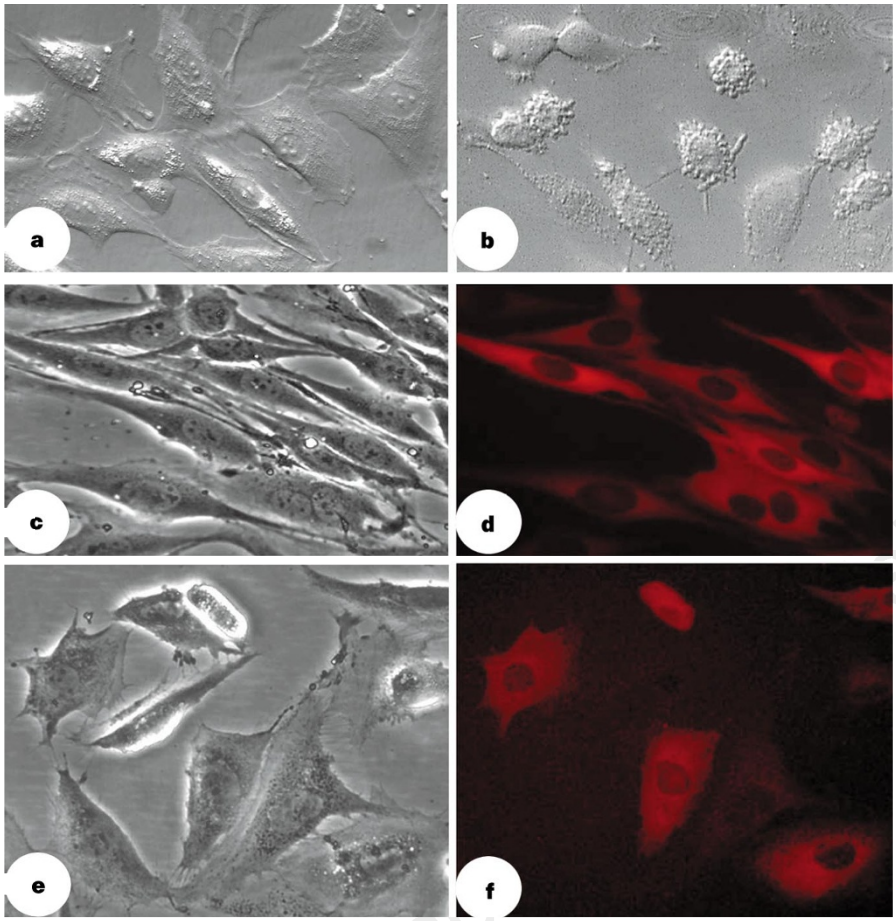

Figure 2 Morphology of microinjected NRK cells. **a**, Control cells. **b**, Wild-type cells injected with 20 μM cytochrome *c*. **c**, Wild type cells preincubated with Z-VAD-fmk for 30 min and co-injected with 20 μM cytochrome *c* and 0.1 μg μl^{-1} TRITC. **d**, Fluorescence micrograph of the same field as that in (c). **e**, Cells overexpressing Bcl-2 co-injected with 20 μM cytochrome *c* and 0.1 μg μl^{-1} TRITC. **f**, Fluorescence micrograph of the same field as that in (e).

and Bcl-2 overexpression, respectively (Fig. 2d, f).

Our results indicate that injected cytochrome *c* can induce apoptosis in various cell types, and that this effect is caspase-dependent. It has been suggested that the role of Bcl-2 protection is restricted to its mitochondrial location, where it prevents leakage of cytochrome *c*[5,6]. Microinjection of cytochrome *c* bypasses the need for release of this protein from mitochondria. However, cells overexpressing Bcl-2 were also protected from the apoptosis induced by injecting cytochrome *c*. This could be explained in several ways.

First, Bcl-2 is not restricted exclusively to the mitochondrial membrane, hence some of its anti-apoptotic property might be based outside this location[1]. Second, Bcl-2 may be a member of the 'apoptosome' complex and it could prevent cell death by direct binding to Apaf-1/Apaf-2/Apaf-3, in a similar way to CED-9's activity on CED-3/CED-4 proteins[9]. Third, it has been reported that cytochrome *c* binds to Bcl-X_L (Ref. 10). Bcl-2 may exert a similar quenching effect outside mitochondria. Fourth, Bcl-2 targets several proteins, including Raf-1, to mitochondria[11]. Therefore when overexpressed, Bcl-2 could possibly act as a transporter for cytochrome *c* back into mitochondria[12].

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Tumour regression after endostatin therapy

It was with great interest that we read the report[1] on the lack of acquired drug resistance to repeated doses of endostatin in experimental cancers in mice. Of particular interest is the unprecedented finding that each tumour type became indefinitely dormant after a varying number of treatment cycles. We believe one explanation for this phenomenon might be found in the clinical observation that, after complete regression of large bulky tumours, rebiopsy of the primary tumour site will frequently show large amounts of fibrosis or scarring.

Pharmacological doses of endostatin, a C-terminal fragment of collagen XVIII with relative molecular mass 20,000, at very low concentrations may be deposited in the extracellular matrix with each cycle of therapy, analogous to amyloid deposits in patients with light-chain disease. The marked shrinkage of the mouse tumours from approximately 250–450 mm^3 to 5–50 mm^3 could effectively increase the local extracellular matrix concentration of endostatin 5–50 fold. Each successive cycle of regrowth followed by treatment and regression could progressively increase the concentration of endostatin in the local extracellular matrix until the concentration is sufficient to inhibit further angiogenesis.

This phenomenon of preferential concentration could explain the observation of a dormant primary tumour while the same tumour type inoculated at a distant site would be uninhibited. The extracellular matrix concentration of endostatin at these distant sites could be several log factors below the concentration at the primary tumour site and insufficient to inhibit tumour-driven angiogenesis.

If this hypothesis is correct, it could mean Boehm *et al.* have serendipitously discovered the ideal way to administer endostatin therapy, delivering a high inhibitory concentration of endostatin at the primary tumour site while keeping the systemic extracellular matrix concentration below the level necessary to inhibit naturally occurring angiogenesis such as wound healing. An assay of the relative concentration of endostatin at the primary tumour site compared to distant tissue concentration should establish if this is the basis for the observed phenomenon.

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