Characterization of a heat-resistant strain of *Tilapia* ovary cells

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Summary

*Tilapia* ovary cells (TO-2) cease to proliferate when moved from normal growth temperature of 31°C to 37°C, and arrest in G₁ and G₂ phases of the cell cycle. The ability of the arrested cells to re-enter the cell cycle when restored to 31°C decreases inversely with time spent at 37°C. A heat-resistant strain, TO-37c, cloned from the surviving fraction of TO-2 after heat treatment, has been found to re-enter the cell cycle with greater facility and to have a higher rate of survival. TO-37c cells have a smaller cell volume than TO-2 and show a distinct morphology at 37°C. Most of the heat-shock proteins (hsp) induced on temperature change were similar, but in TO-37c the decline in the synthesis of a 27×10³ Mr hsp was faster and a 37°C-specific 60×10³ Mr hsp was missing. Ultraviolet (u.v.) sensitivity was slightly affected if heat treatment was given after irradiation. However, when cells were preheated and then u.v. irradiated, the u.v. sensitivity increased sharply for TO-2 cells but not for TO-37c.

Key words: fish cells, thermoresistance, G₁,G₂ arrest, u.v. sensitivity.

Introduction

In previous papers we have reported that cell lines TK and TO-2, established from the warm-water fish *Tilapia* can be grown readily at various temperatures from 18°C to 34°C (Chen & Yew, 1988; Chen et al. 1988). The cells exhibited reasonable resistance towards ultraviolet radiation, which is unusual among fish cells. In general, fish cells lack excision repair of pyrimidine dimers (Mano et al. 1980, 1982; Regan et al. 1972; Shima et al. 1981; Shima & Setlow, 1984). Although *Tilapia* cells are more u.v.-resistant than Chinese hamster ovary (CHO) cells, they have a similar level of DNA excision-repair (Chen & Yew, 1988; Yew & Chang, 1984). The effects of ultraviolet light (u.v.) on the rate of DNA replication and daughter-strand initiation and elongation in TO-2 are also qualitatively similar to those of CHO cells, but higher u.v. doses are required to achieve the same quantitative results as in CHO (Chen & Yew, 1988; Yew & Chang, 1984).

Changes in environmental temperature induce synthesis of various proteins. The specificities of these proteins with respect to temperature variation and the kinetics of protein synthesis have also been reported in *Tilapia*. The major heat shock protein (hsp) groups induced were the 100, 87, 70, 60, 44 and 27K (K = 10³ Mr) proteins. Among them the 27K hsps are probably fish-specific (Chen et al. 1988) or even tissue-specific (C. Ku, personal communication). The 60K and 44K hsps are induced at 37°C but not at higher temperatures.

During these experiments, we found that when cultures of TO-2 cells were kept at 37°C for a few days they were, in the main, unable to grow and form colonies at 31°C. One exception is a variant clone, TO-37c, which after 37°C treatment shows a moderate plating efficiency at 31°C. In this paper we have characterized the growth ability of these spontaneous variant cells at elevated temperature, their protein synthesis after shifting from 31°C to 37°C, and their response to u.v. radiation as well as excision repair of pyrimidine dimers at 37°C.

Materials and methods

Cells and culture conditions

The TO-2 cell line was originally established from the ovary of adult *Tilapia*, a hybrid of *Tilapia mossambica* and *T. nilotica* (Chen et al. 1983). The cells were maintained in Leibovitz’s L-15 medium (Flow) supplemented with 10% foetal bovine serum (GIBCO, Santa Clara, CA). The cultures were maintained in a humidified incubator at 31°C.

Cell cycle and cell volume analysis

Cell cycle and cell volume were analysed by a Becton Dickinson FACStar Flow Cytometer. For cell cycle analysis the cells were fixed in 70% ethanol and stained with propidium iodide.

Heating

Heating of monolayers of cells was carried out in precalibrated...
water baths. Monolayers of cells in parafilm-sealed Petri dishes or flasks were immersed completely in a hot-water bath for acute heat shock treatments. The temperature of the water bath was controlled to within ±0.1 deg. C. For long-term thermal exposure at 37°C, the cultures were shifted to precalibrated incubators. The temperature of the incubators was controlled to within ±0.3 deg. C.

Survival assay
Cells were heated or u.v. irradiated in monolayers at the exponentially growing phase. After each treatment cells were trypsinized, counted with a Coulter counter or a haemocytometer, serially diluted, plated into 60 mm Petri dishes and incubated at 31°C for 7 days to allow single surviving cells to form colonies. Colonies were rinsed once with phosphate-buffered saline (PBS) and stained with 5% Crystal Violet dissolved in 70% ethanol. The fixed and stained colonies were rinsed with tap water and counted under a dissection microscope. Only those colonies with more than 50 cells were scored. The plating efficiency for control untreated TO-2 cells was above 80%.

Protein labelling
Cells were labelled with 100 μCi of [35S]methionine (specific activity 1200 mCi/mmol) in methionine-free medium for 8 h immediately after shifting to elevated temperature (37°C). L-15 medium without methionine was custom made from Culture Medium Facility, University of California, San Francisco. MEM medium without methionine was made according to NIH.

Two-dimensional gel electrophoresis
The details of two-dimensional gel electrophoresis for the analysis of heat-induced protein synthesis have been described. The first dimension was isoelectrofocusing (O'Farrell, 1975) and the second dimension was sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970).

Results
Selection of heat-resistant strain TO-37c
TO-2 cells were allowed to grow to subconfluence at 31°C. The culture was then moved to 37°C for 7 days. Only a few cells survived. The cultures were moved back to 31°C, and after a week some large colonies formed. One colony was scraped off and transferred to a new flask, and the cells were allowed to grow to subconfluence and heat-treated as before. A surviving colony was cloned. When the new culture reached subconfluence once more, it was subjected to acute heat shock at 43°C for 30 min. Only two clones survived, one of them was TO-37c.

Growth properties at 37°C
TO-37c and TO-2 proliferated at a similar rate at 31°C (doubling time 10 h), but both stopped growing at 37°C (Fig. 1). Since the number of cells plated was 10⁵ and the size of the dish was 60 mm in diameter, the cell density was far below confluency, and the stop in growth should be due to the inhibitory effect of 37°C heating. The rates of proliferation of TO-2 at 31°C and 37°C have been reported (Chen et al. 1988). When kept at 37°C, the cell volumes expanded. Morphologically the cells are similar in appearance at 31°C except that TO-37c has a smaller volume than TO-2. Flow cytometry confirmed this size difference with a ratio of 1:1.5; however, when both cells are incubated at 37°C the volume increases as incubation time is prolonged (Fig. 2), the morphology at 37°C becomes distinct from that at 31°C, and the difference between TO-2 and TO-37c is apparent (Fig. 3).

The cell cycle distribution of TO-2 and TO-37c was also measured by flow cytometry. The DNA histograms of both strains were characterized by a very short S-phase; 10% of the total population for TO-2 and 13% for TO-37c, and the generation time for each was about 10 h. These results were confirmed by measuring the pulse-labelling index for S-phase with [3H]thymidine. When the temperature was raised to 37°C, the duration of the S-phase of TO-2 increased to 23% after 48 h but remained

![Fig. 1. Growth rate at 37°C. Cells were plated at 10⁵ cells per dish and incubated at 31°C for 24 h, then moved to a 37°C incubator. Plates were taken out at 24-h intervals and cell numbers were counted. Each point is the average of four plates. (●) TO-2; (■) TO-37c.](image1)

![Fig. 2. Change in cell volume at 37°C. Samples were taken at various times after incubation at 37°C. They were fixed immediately with 70% ethanol and stained with propidium iodide. The relative volumes of cells were measured by a flow cytometer. (●) TO-2; (■) TO-37c.](image2)
Fig. 3. Change in morphology at 37°C. Photographs were taken under a phase-contrast microscope at ×100. A, TO-2, control; B, TO-2, 37°C, 54 h; C, TO-2, 37°C, 74 h; D, TO-37c, control; E, TO-37c, 37°C, 54 h; F, TO-37c, 37°C, 74 h.
Induced protein synthesis at 37°C

When TO-2 cells were shifted from 31°C to 37°C, a group of proteins was preferentially synthesized (Fig. 6). The most prominent of the newly synthesized groups were around 27K, 87K and 100K, these proteins were also synthesized after acute (43°C) heat shock and were known as heat shock proteins (hsp3). The hsp3 appeared within the first 8 h, then gradually decreased in quantity. Two proteins of 60K and 44K, which were only synthesized at 37°C, were found to be at the maximum level between 8 and 16 h after temperature change (Chen et al. 1988). The two-dimensional pattern of the proteins synthesized by TO-37c at 31°C were qualitatively similar to that of TO-2; all hsp3 were synthesized in large quantities during the first 8 h after changing to 37°C. During the second 8-h period of synthesis, the synthesis of the 27K group was completely turned off, while the synthesis of 87K and 100K proteins continued. Of the two proteins synthesized in TO-2 cells only at 37°C, the 60K protein was not synthesized in TO-37c cells, only the 44K species appeared.

Heat shock at 43°C

TO-37c was selected from TO-2 first by chronic heating at 37°C and then reselected by acute heat shock at 43°C for 30 min. The thermal survival responses after 15 min of treatment at 43°C showed a 10-fold improvement in TO-37c compared with that in TO-2 cells (Fig. 7).

u.v. killing at 37°C

To test whether 37°C would enhance cell killing by u.v. radiation, the cells were irradiated at 31°C, then shifted to 37°C and incubated for 2 or 4 h. Afterwards they were moved back to 31°C for 7 days to permit colony formation. The results are shown in Fig. 8. At 31°C TO-2 and TO-37c have similar u.v. sensitivity, and the 2-h post-u.v. incubation at 37°C did not have any effect (results not shown). After 4 h post-u.v. incubation at 37°C the u.v. sensitivity of both cells increased slightly but there was no difference between them. The thermal killing effect on unirradiated TO-2 cells (Fig. 5) has been normalized in the plot in Fig. 8.

When cells were heated at 37°C for 2 h before u.v. irradiation and subsequently allowed 2 h to repair at 37°C, the effects on their survival were very different. TO-2 showed a sharp increase in u.v. sensitivity while that of TO-37c increased only slightly.

Discussion

TO-2 cells are characterized by their tolerance to u.v. and chemicals (Chen & Yew, 1988; Yew & Chang, 1984; Wang & Yew, 1987). This is very unusual among fish cells, for most of them do not express excision repair after DNA damage and are very sensitive to u.v. irradiation (Mano et al. 1980, 1982; Regan et al. 1972; Shima et al. 1981; Shima & Setlow, 1984). We find that TO-2 cells have a doubling time of 10 h, but spend only 1 h in S-phase. Since u.v. sensitivity varies with the phase of the cell cycle, the early S-phase being the most sensitive

unchanged for TO-37c. Most of the cells were arrested in G1 and G2 (Fig. 4).

To test whether prolonged arrest at 37°C is cytotoxic, the cells were plated first at 31°C, for 20 h to ensure attachment before shifting to 37°C for various periods. Cells were returned to 31°C to assess their ability for clonal growth. The results are shown in Fig. 5, and indicate that if TO-2 cells are kept at 37°C, they are rapidly killed by heat shock. TO-37c cells were also affected but to a lesser extent (Fig. 5).

Fig. 4. Redistribution of cell cycle at 37°C. S phase population was obtained from [H]thymidine pulse-labelling experiments. The G1 and G2/M populations were calculated from DNA histograms produced by a flow cytometer. Filled symbols, TO-2; open symbols, TO-37c; S-phase (●, △); G1-phase (●, ○); G2/M-phase (■, □).

Fig. 5. Thermal killing at 37°C. Cells were plated at 31°C overnight for attachment. They were moved to a 37°C incubator for various periods and then moved back to 31°C for a week to allow colony formation. Each point is an average of four plates. (●) TO-2; (■) TO-37c.

Fig. 6. Protein synthesis at 37°C. The early S-phase population was obtained from [H]thymidine pulse-labelling experiments. The G1 and G2/M populations were calculated from DNA histograms produced by a flow cytometer. Filled symbols, TO-2; open symbols, TO-37c; S-phase (●, △); G1-phase (●, ○); G2/M-phase (■, □).
Fig. 6. Proteins synthesized at 37°C. TO-37c cells were labelled with [35S]methionine for 8 h for the indicated periods and at the temperatures shown. The cells were lysed and prepared for two-dimensional acrylamide gel electrophoresis as described in Materials and methods. Note that hsps 27b and 27c were not synthesized after 8 h, and hsp 60 was not synthesized at all.
period (Collins & Johnson, 1979; Collins & Waldren, 1982; Downes et al. 1979, 1982), if a cell spends less time in S-phase, it should be more u.v.-resistant. In addition, although TO-2 cells have little excision repair ability their post-replication repair is very efficient (Chen & Yew, 1988), and this may contribute largely to their u.v. tolerance. Experiments on synchronized cells are in progress to elucidate this point.

A heat-resistant line, TO-37c, has been selected from TO-2 by prolonged incubation at 37°C, followed by brief heat shock at 43°C. There were differences in morphology and cell volume, but cell cycle distributions are the same for both cell lines. Incubation at 37°C appeared to hold a great number of cells in the 4c state, presumably in G2. This phenomenon has been reported by Chen & Wang (1982) and Zaitsu & Kimura (1985, 1988) on temperature-sensitive mutants of CHO and rat fibroblasts. These workers also report that prolonged G2 holding is not reversible and results in cell killing. The rodent cells reported to possess this unusual property were selected from wild-type cells, which proliferated normally at the non-permissive temperature. In contrast, Tilapia ovary cells, both wild-type (TO-2) and heat-resistant strains showed G1 and G2 holding at 37°C. The fact that TO-37c was able to reverse the G2 block better than TO-2 could be due to the presence of certain protein factors that fulfilled the serum requirement as suggested by the experiments of Zaitsu & Kimura (1988).

There are few differences in induced protein synthesis between TO-2 and TO-37c when changing from 31°C to 37°C. The kinetics of induction and decay of protein synthesis might be faster in TO-37c, but we have no evidence to suggest that these features are related to the heat-resistant phenotype. The only protein that did not appear in TO-37c was the 60K hsp, which is one of the 37°C-specific inductions in TO-2.

Since TO-37c cells can tolerate 37°C chronic heating for a longer time than the wild-type TO-2, the question of whether they could survive another stress-inducing agent, such as u.v., better at this temperature is of interest. Moreover, we were curious to learn whether the production of heat shock proteins would be beneficial or harmful to the cells' tolerance of u.v. damage. For TO-2 cells, when the temperature was raised after the u.v. challenge, there was only a slight effect on u.v. sensitivity. However, when heat was given before u.v. there was a sharp increase in sensitivity. In contrast, the u.v. sensitivity of TO-37c cells was only slightly affected by heat whether it was given before or after the challenge. The incision rates at pyrimidine dimers induced by u.v. in TO-2 and a temperature-resistant line TO-37E have been reported (Chen & Yew, 1988); the incision rates peak at 31°C but decline sharply as temperature rises. We found that TO-37c cells also have the same level in

Fig. 8. u.v. killing at 37°C. Cells were plated and incubated at 31°C overnight for attachment. u.v. irradiation was performed with cells in PBS, but in complete medium during heat treatment. A. Cells were u.v. irradiated and incubated at 37°C for 4h; B, cells were preheated at 37°C for 2h, then irradiated with u.v. and further incubated for 2h at 37°C. Surviving fractions were calculated from colony formation at 31°C. Filled symbols, TO-2; open symbols, TO-37c. (○, □) Control; (■, ■) 4h post-u.v. heating; (▲, △) 2h pre-u.v. and 2h post-u.v. heating.
excision repair and behave similarly with variation in temperature to those two cell lines (results not shown). We conclude that excision repair is not influenced by heat shock and therefore that changes in u.v. sensitivity of Tilapia cells are not attributable to excision repair. The difference in the heat response of u.v. sensitivity of TO-2 and TO-37c indicates that at least one major cellular process other than excision repair is temperature sensitive in TO-2, but tolerant in TO-37c.

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References


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