Journal of Cellular Biochemistry 75:327–337 (1999)

Effects of Hyperthermia on the Cytoskeleton and Focal Adhesion Proteins in a Human Thyroid Carcinoma Cell Line

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Abstract Hyperthermia is reported to act as a sensitizer to chemotherapeutic drugs in the treatment of cancer. Thyroid follicular carcinoma were used to elucidate the effects of hyperthermic treatment (41–43°C) on cell morphology, cytoskeleton, and the focal adhesion complex. The critical temperature that resulted in inhibition of cell proliferation as the cell number in the same area did not increase over a 23 h time course and irreversible changes in cell morphology was 42–43°C. An immunofluorescence study on heat-treated cells (43°C, 1–5 h) demonstrated that depolymerization of actin filaments, intermediate filaments, and microtubules accounted for the rounding-up of cells and detachment from the substratum. Characteristic staining patterns for integrin αv, focal adhesion kinase, and vinculin were noted in untreated cells, but the immunoreactive intensities for these proteins became weaker with time of heat treatment. Anti-phosphotyrosine staining revealed less immunoreactivity in the focal adhesions in treated cells compared with control cells. The disappearance of integrin αv from the cell surface may result in inhibition of integrin-mediated activation of focal adhesion kinase, which results in dephosphorylation of focal adhesion components and its disassembly. These results indicate that hyperthermia induces disruption of integrin-mediated actin cytoskeleton assembly and, possibly, of other integrin-mediated signaling pathways. J. Cell. Biochem. 75:327–337, 1999. © 1999 Wiley-Liss, Inc.

Key words: thyroid follicular carcinoma cells; hyperthermia; integrin; focal adhesion; cytoskeleton

Because of its clinical use in radiotherapy and chemotherapy of cancers, the effects of hyperthemia (41–45°C) on mammalian cells have been extensively studied during the past decade [Overgaard, 1989; Storm, 1993; Schepotin et al., 1994]. Acute heat treatment induces the heat shock response, which helps the cells to survive. These responses include effects on gene regulation, chromatin organization, synthesis of DNA, RNA and proteins (including heat shock proteins), cytoskeletal reorganization, and cellular Ca^{2+}-homeostasis [Nover, 1984; Burdon, 1986].

The response of the cytoskeletal system to hyperthermia varies depending on the cell type and heat dose. Not all three cytoskeletal elements (microtubules, intermediate filaments, and actin filaments) are affected simultaneously during heat treatment, with sometimes one or two being affected, while the other(s) does not undergo reorganization [Lin et al., 1982; Glass et al., 1985; Wachsberger and Coss, 1990]. In addition to its effect on microtubule polymerization, hyperthermia also disrupts microtubule organization center and mitotic spindles [Coss et al., 1982]. In some cases of heat shock, the vimentin filaments form perinuclear aggregates [Biessmann et al., 1982; Wachsberger and Coss, 1990]. A rapid loss of stress fibers in Chinese hamster ovary cells has been reported after immersion in a 45°C water bath [Coss et al., 1982; Glass et al., 1985].

The structural integrity of the actin cytoskeleton is maintained by integrin-based signal transduction at focal adhesions (FAs) [Juliano and Haskill, 1993], and actin depolymerization prevented phosphorylation of focal adhesion kinase (FAK) [Shattil et al., 1994]. Heat induces the shedding of αvβ3 integrin, but not α3β1

Grant sponsor: National Science Council, Republic of China; Grant number: NSC 88–2314-B-002–058; Grant sponsor: National Taiwan University Hospital; Grant number: NTUH 88 S1022.

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Received 3 March 1999; Accepted 26 April 1999

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integrin, from the membrane surface of A549 human lung tumor cells [Majda et al., 1994]. The effect of hyperthermia on integrin signal transduction therefore deserves further study.

Hyperthermia affects cell adhesion and reduces tumor growth in experimental animals and in human cancer patients [Green et al., 1990; Shen et al., 1987], but increases bone and lung metastasis of osteogenic sarcoma in dogs [Lord et al., 1981]. Since thyroid tumors or recurrent thyroid tumors are located superficially, within 1–2 cm of the skin, biotechnical application of heat is feasible. In order to obtain a better understanding of the effects of hyperthermia on thyroid follicular carcinoma, we used CGTH W-2 cells, human thyroid carcinoma cell line, as a model in a heat shock study. Cytoskeletal changes, including those involving actin filaments, vimentin filaments, and microtubules, were examined using immunofluorescence techniques. In addition, effects on the distribution of the focal adhesion (FA) components, vinculin, and FAK, and their transmembrane counterpart, integrin, were also examined.

MATERIALS AND METHODS

Cell Culture

The CGTH W-2 cell line, derived from a metastatic thyroid follicular carcinoma from a Chinese patient, was a generous gift from Dr. Jen-Der Lin [Lin et al., 1996]. The cells were grown in a 5% CO_2 humidified atmosphere at 37°C and RPMI containing 10% fetal calf serum, 1 µM sodium pyruvate, penicillin (100 U/ml), and streptomycin (10 µg/ml; pH 7.2–7.4). For time-course studies, cells were plated onto etched grid coverslips (Belloco Glass Inc., Vineland, NJ) to facilitate the relocation of cells of interest.

Hyperthermia Treatment

CGTH W-2 cells were incubated in an incubator of 5% CO_2 at 37°C, 41°C, 42°C, or 43°C for 1–5 h. After heat shock, the cells were allowed to recover in an incubator of 5 % CO_2 at 37°C for 2 or 18 h. For time-course studies, phase images were taken at various intervals.

Cell Attachment Inhibition Assay

CGTH W-2 cells were plated in 96-well plates and incubated with a 1/10 dilution of antibodies (monoclonal mouse anti-human αvβ3 antibody, monoclonal rat anti-human α6 antibody and rabbit anti-human α3 antiserum [Chemicon, Temecula, CA]) for 8 h. Phase images were recorded at 30 min intervals.

Immunofluorescence Microscopy

Mouse monoclonal antibodies directed against β-tubulin, vimentin, vinculin, and phosphotyrosine were purchased from Sigma (St. Louis, MO). Mouse monoclonal anti-FAK antibodies and polyclonal rabbit anti-integrin αv antiserum were purchased from Transduction Lab. (Lexington, NJ). Antibody reactions were performed at 37°C and all other manipulations at room temperature. Cells grown on coverslips were washed briefly in PBS, fixed in 10% formalin in PBS for 10 min, and permeabilized using 0.1% Triton X-100 in PBS for 10 min. Nonspecific binding sites were blocked by treatment with 5% non-fat milk in PBS for 15 min. Primary antibodies used in this study was listed in Table 1. For single labeling, after a PBS wash, the cells were incubated with diluted primary antibodies (Table 1) for 1 h, then with an appropriate second antibody (1/50 diluted FITC-conjugated goat anti-mouse IgG or anti-rabbit IgG antibodies; Sigma) for 1 h. Double-labeling for actin and vinculin was performed by incubation with mouse anti-vinculin antibody, followed by incubation first with 1/50 diluted biotinylated anti-mouse IgG antibody

TABLE I. List of Primary Antibodies

Cytoskeletal protein	Detecting probe
Actin	FITC-phalloidin (5 units/ml)
Vimentin	1:100 mouse monclonal anti- vimentin
Tubulin	1:40 rabbit anti-tubulin
Focal adhesion complex associated proteins	
Vinculin	1:200 mouse monoclonal anti-vinculin
Focal adhesion kinase	1:50 mouse monoclonal anti-focal adhesion kinase
Photophotyrosine	1:50 mouse monoclonal mouse-phosphotyrosine
Integrins	
$\alpha_v\beta_3$	1:10 mouse anti-human $\alpha_v\beta_3$
α_6	1:10 rat monoclonal anti-human integrin α_6
α_3	1:10 rabbit anti-human integrin α_3

(Vector, Burlingame, CA) for 1 h, then with 1/50 diluted avidin-Texas red (Vector) for 30 min. After a 5-min wash with PBS, the cells were incubated with FITC-conjugated phalloidin (Sigma) for 30 min. After extensive PBS washes, the cells were mounted on a slide using a mounting medium containing 2% n-propyl gallate, 60% glycerol in PBS, pH 8.0, and sealed with a nail polish. Staining of the cells was examined using a Reichert Polyvar 2 microscope (Leica, Vienna, Austria), equipped for epifluorescence, and images were taken using Kodak T-max 400 film.

RESULTS

Alterations of Cell Morphology After Heat Treatment

To determine if heat treatment causes changes in cell morphology, cells were cultured on a grid-coverslip and their phase images recorded at various intervals during the experiment. CGTHW-2 cells have a doubling time of 18 h at 37°C [Lin et al., 1996]. The untreated cells duplicated during the 23 h study; the cell number per unit square in 0 h (Fig. 1A) doubled after every 23 h (Fig. 1B,C). After exposure to heat shock at 41°C, the cells appeared normal, except that they exhibited several slender processes after 5 h of treatment (Fig. 1C,E, control A). After a recovery period of 2–18 h, these processes retracted, and the morphology of the cells returned to normal; however, the cell number did not increase after 23 h (Fig. 1I, control A). In contrast, when heated to 42°C, the cells became rounded-up after 3 h (Fig. 2D, control B) and detached from the substratum after 5 h (Fig. 2F); when allowed to recover at 37°C for 2–18 h, only a few assumed a normal shape (Fig. 2H,J). Exposure to a temperature of 43°C resulted in more severe morphological changes, with shrinkage of the cell body and cell processes being noted (Fig. 3B,C, control A), this effect being irreversible even after a recovery period of 2–18 h (Fig. 3D,E). Heat treatment at 42–43°C inhibited the increase in cell number in total 23 h study as shown in Fig. 2J (control 2A) and Fig. 3E (control 3A).

Effect of Hyperthermia on Focal Adhesion Proteins and Cytoskeleton

FA are macromolecular complexes that link actin filaments to clusters of cell substrate adhesion receptors (integrins and proteoglycans) [Burridge et al., 1988]. In this study, FAs were identified by immunofluorescent staining for vinculin or FAK. In untreated cells, in addition to junctional staining, vinculin was present as oval plaques that were evenly distributed near the ventral plasma membrane (Fig. 4B); these are the insertion sites for actin filaments (Fig. 4A). In control cells, anti-vinculin antibody revealed two groups of FAs, one perinuclear (central) and the other at the cell periphery (peripheral). No significant change in vinculin or actin staining was seen in cells maintained at 43°C for 1 h (Fig. 4C,D). However, a longer incuba-

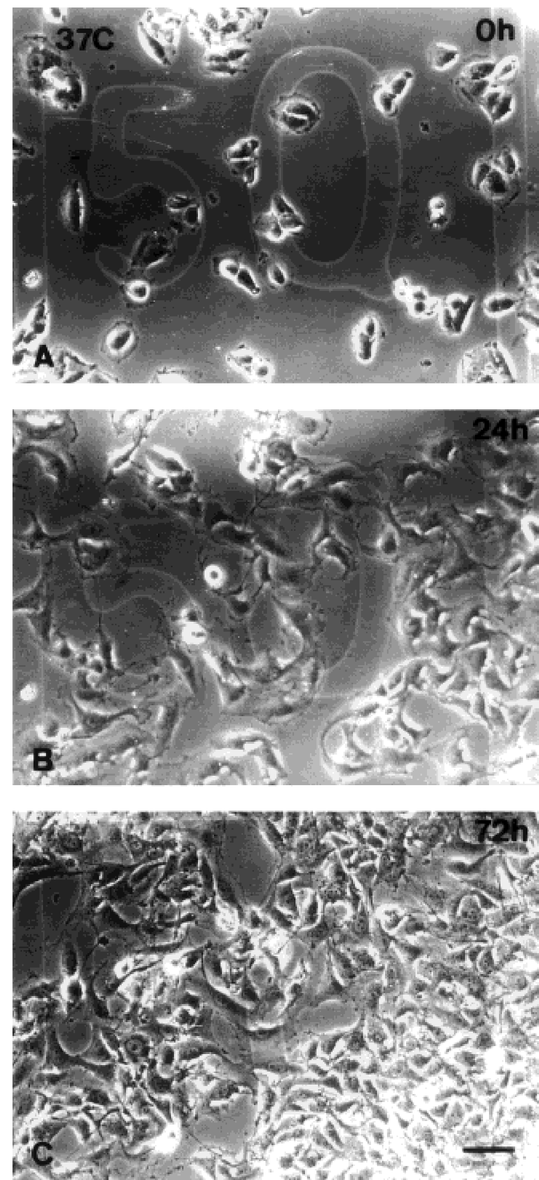

Fig. 1. Growth pattern of CGTH W-2 cells at 37°C. **A**: 0-h culture. **B**: 24-h culture. **C**: 48-h culture. Scale bar = 50 μm.

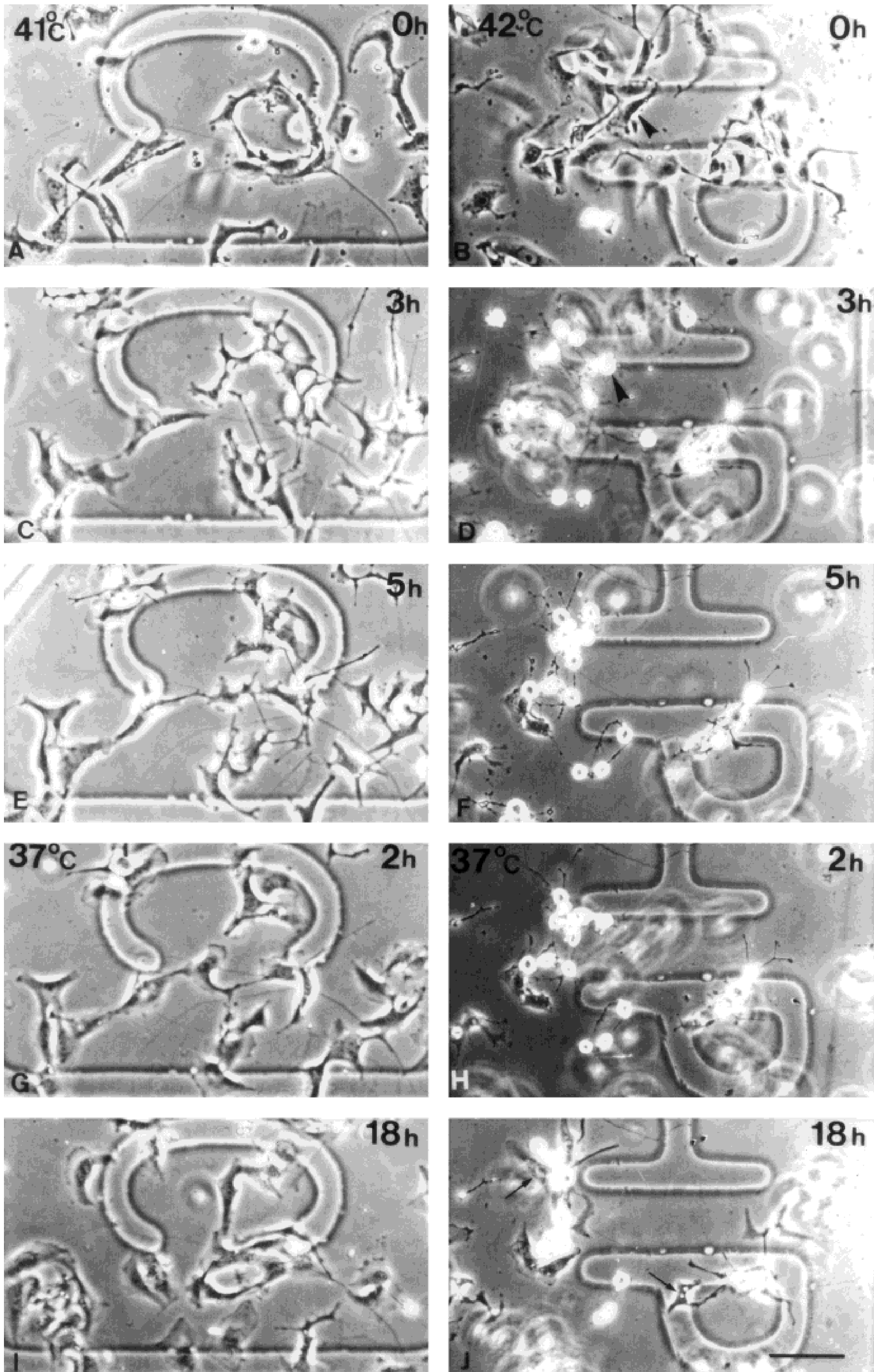

Fig. 2. Morphological changes in CGTH W-2 cells during 41°C and 42°C heat shock. Cells were heat shocked at 41°C for 0 (**A**), 3 (**C**), or 5 h (**E**), then allowed to recover at 37°C for 2 (**G**) or 18 h (**I**). The cells become extended and change their relative position with time of incubation. **B,D,F**: Cells heat shocked at 42°C for 0, 3, or 5 h, respectively. Cell retraction is noted after 3 h of 42°C treatment (D). **H,J**: cells heated at 42°C and then allowed to recover at 37°C for 2 or 18 h, respectively. Only few cells (arrows in J) become flattened after 18 h recovery. Scale bar = 100 μm.

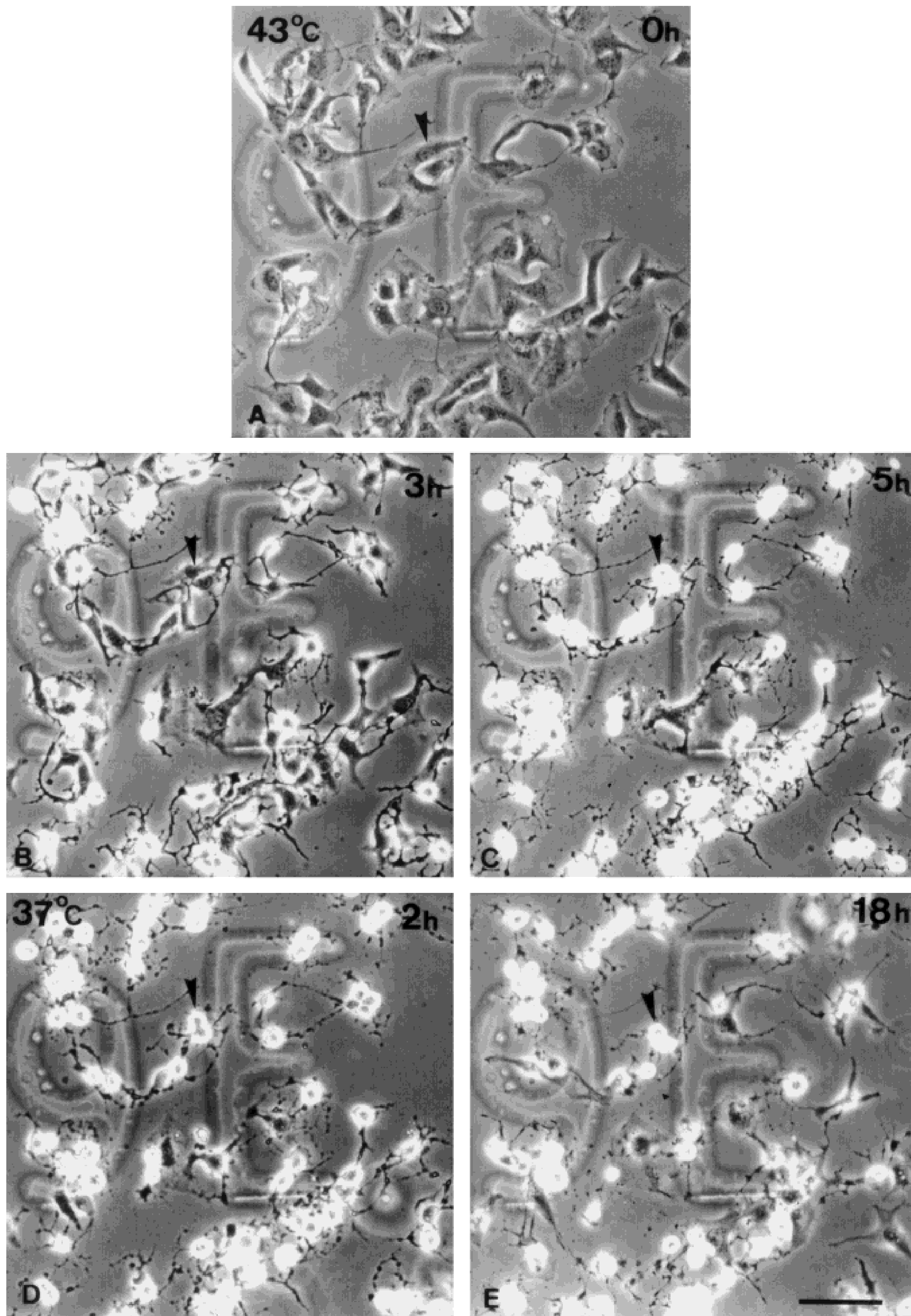

Fig. 3. Morphological changes in CGTH W-2 cells after 43°C heat shock. Cells were incubated at 43°C for 0 (**A**), 3 (**B**), or 5 (**C**) h, then allowed to recover at 37°C for 2 (**D**) or 18 h (**E**). The arrrow-head indicates the same cell. Cells show shrinkage and retraction after 3 h of treatment (B) and most fail to assume their original shapes (E) after 18 h of recovery. Scale bar = 100 μm.

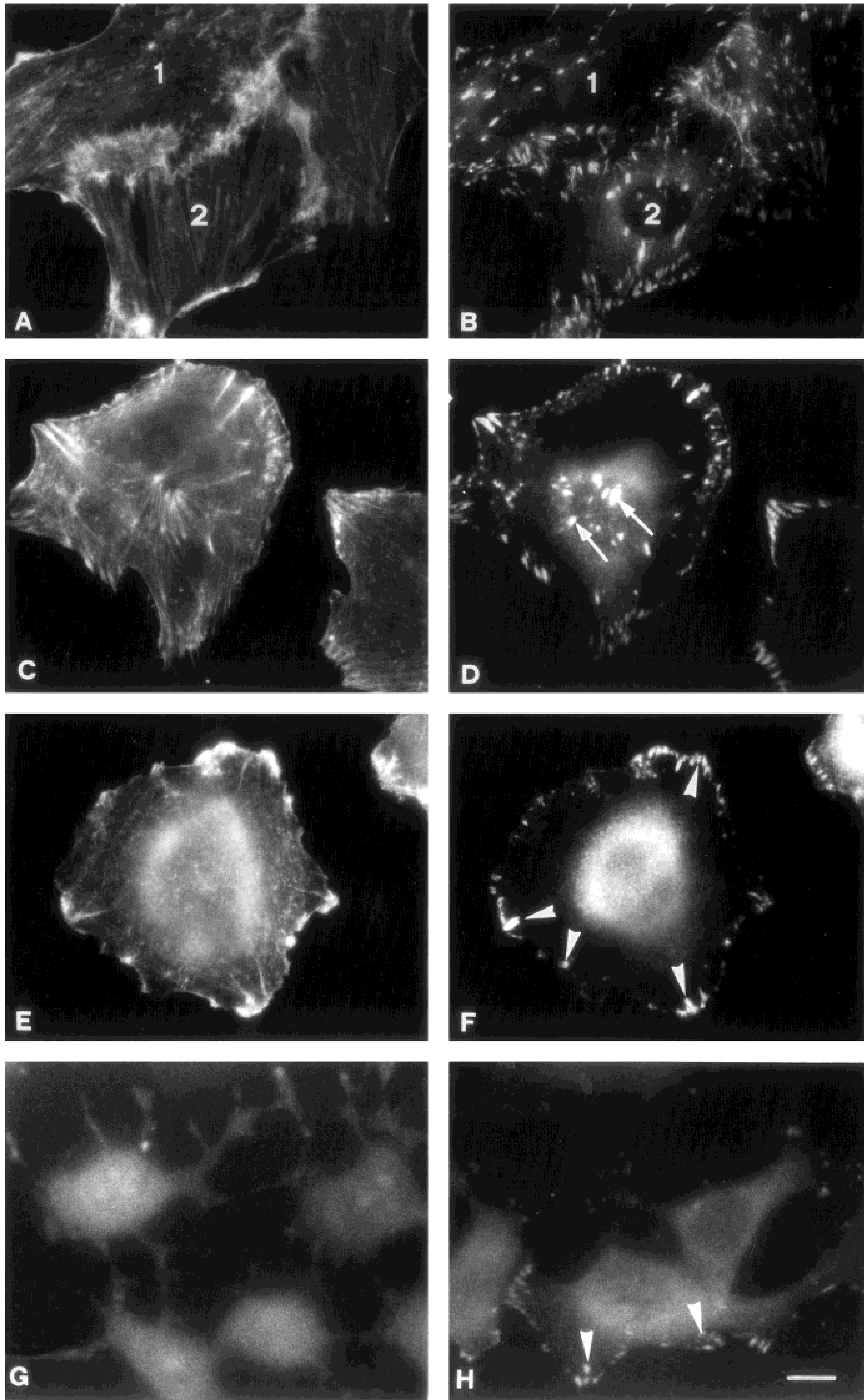

Figure 4.

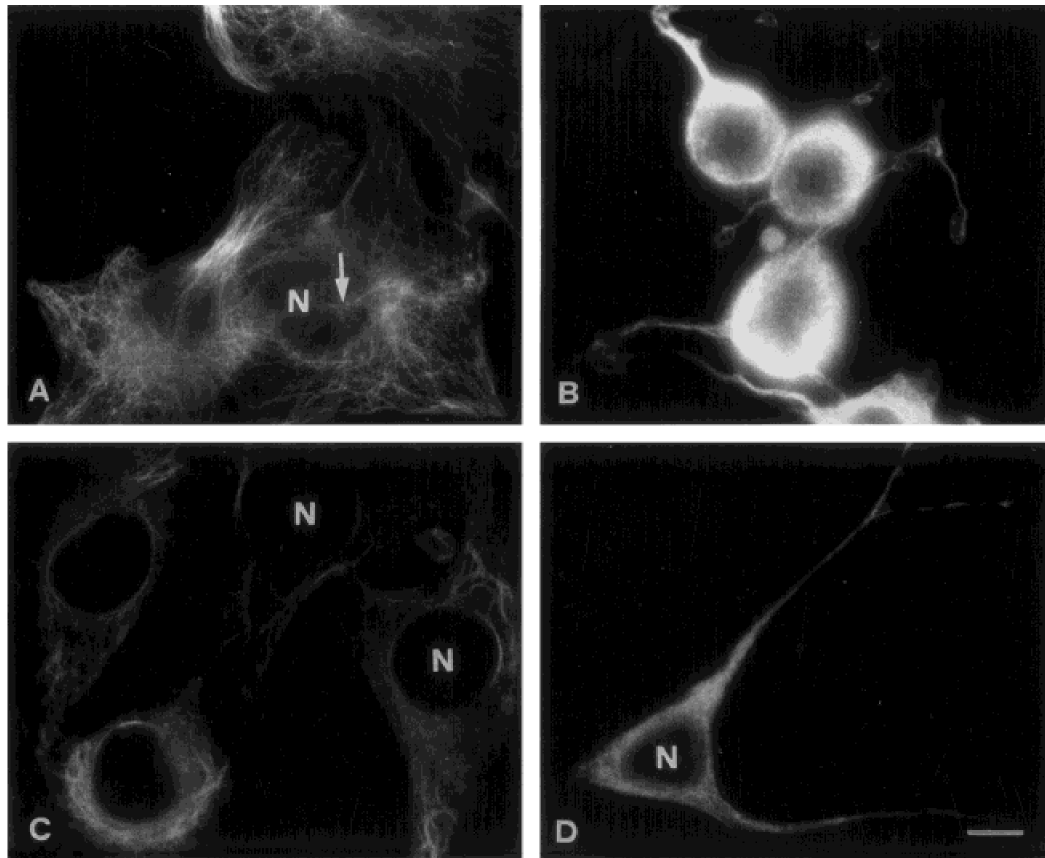

Fig. 5. Effect of heat shock on the distribution of microtubules and vimentin filaments in CGTH W-2 cells. In control cells, the microtubules radiate from an organization center (arrow; **A**). Tubulin staining is diffuse after heat shock at 43°C for 5 h (**B**). In control cells, there is a filamentous network of vimentin filaments (**C**). Staining is amorphous in heat-treated cells (**D**). Scale bar = 10 μm.

tion period (3 h) induced progressive depolymerization of actin filaments (Fig. 4E) and the disassembly of the central FA, which was revealed as diffuse staining, but the peripheral FA remained intact (Fig. 4F, arrowheads). Peripheral FAs were eventually disassembled after 5 h at 43°C (Fig. 4G,H). As regards the other cytoskeleton elements, microtubules were arranged radially in the cytoplasm of untreated cells (Fig. 5A); but were depolymerized in cells treated at 43°C for 5 h (Fig. 5B). Vimentin filaments appeared as a filamentous network under normal conditions (Fig. 5C), but became fewer in number and were depolymerized after treatment at 43°C for 5 h (Fig. 5D).

Integrin Expression and FAK Distribution

Normal thyroid epithelial cells express integrin $\alpha3\beta4$, whereas neoexpression of integrin $\alpha6\beta4$ is seen in human thyroid carcinoma cells [Serini et al., 1996]. In this study, only the presence of integrin $\alpha v\beta3$ was confirmed using the cell attachment assay or immunofluorescence staining, whereas anti-integrin $\alpha3$ and anti-integrin $\alpha6$ failed to react with these cells (data not shown). On incubation with anti-integrin $\alpha v\beta3$ antibody, CGTH W-2 cells became detached from the substratum (Fig. 6). In control cells, the integrin αv staining pattern was similar to that of FAs (Fig. 7A). Treatment at 43°C for 2 h had no effect on integrin distribu-

Fig. 4. Effect of heat shock on the distribution of actin and vinculin in CGTH W-2 cells. A–H: Double-staining for actin (FITC-phallotoxin) and vinculin, respectively. In control cells in the control group, actin bundles (**A**) are associated with vinculin-positive focal adhesions (**B**) at their termini. Cells were heat shocked at 43°C for 1 h (**C,D**), 3 h (**E,F**), or 5 h (**G,H**). With increasing time of heat shock, actin bundles depolymerize (E,G), concomitantly with the disappearance of vinculin staining (F,H). The arrows and arrow-heads indicate the central and peripheral focal adhesions, respectively. Scale bar = 10 μm

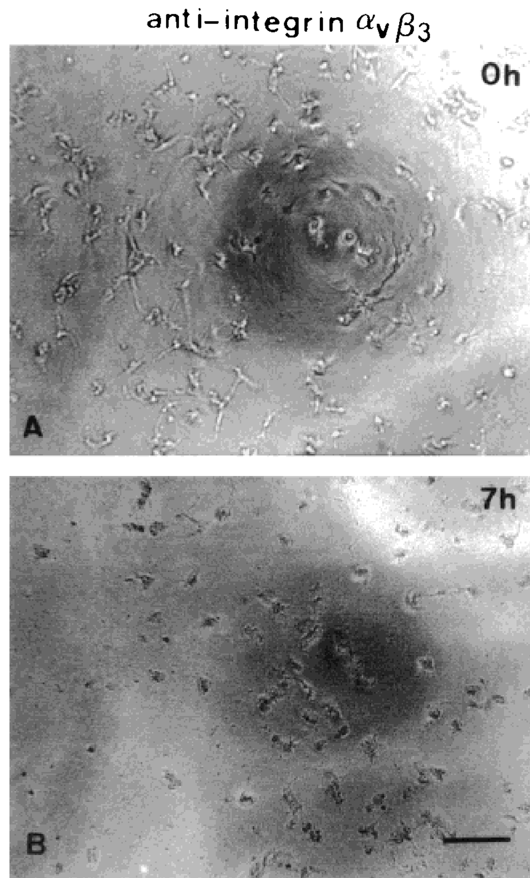

Fig. 6. Detachment of CGTH W-2 cells by incubation with anti-integrin antibody. **A:** Before addition of antibody. **B:** Seven h after incubation with anti-integrin αvβ3 antibody. Cells become rounded-up and detached from the substratum. Scale bar = 100 μm.

tion (Fig. 7C), whereas treatment at 43°C for 3 and 4 h resulted in more diffuse staining for integrin αv (Fig. 7E,G). Since FA formation is dependent on the clustering of integrin, and FAK phosphorylation which triggers stress fiber formation [Defilippi et al., 1997], we also examined the effect of hyperthermia on FAK. FAK, a tyrosine kinase, is localized in FAs [Burridge et al., 1992]; this was confirmed in control cells (Fig. 7B). In response to hyperthermic treatment at 43°C for 2–4 h, FAK staining was lost (Fig. 7D,F,H), being occasionally found in the perinuclear cytoplasm of rounded cells (Fig. 7H). In order to examine the degree of tyrosine phosphorylation of the cells, anti-phosphotyrosine immunostaining was used. In normal cell, phosphotyrosine staining was concentrated in the FAs (Fig. 8A), whereas with increasing time of heat treatment (2–4 h), staining was seen as smaller dots (Fig. 8B,C), then became diffuse (Fig. 8D).

DISCUSSION

In this study, we have shown that, at a critical temperature of 42–43°C, CGTH W-2 thyroid carcinoma cells first became rounded-up, then detached from the substratum, and that the attached, rounded-up cells were unable to return to a normal cell morphology when allowed to recover at 37°C for 18 h. In addition to cell adhesion, cell proliferation was also inhibited by hyperthermia. In adherent cells like CGTH W-2 cells, adhesion to the substratum is required for cell proliferation, a common feature in most mammalian cells [Guadagno et al., 1993]. In addition, since cell adhesion is involved in the cyclin A-mediated regulation of cell proliferation of attached cell lines [Guadagno et al., 1993], the poor adhesion of CGTH W-2 cells apparently inhibited proliferation after heat shock.

The disruption of all three cytoskeletal components (actin filaments, microtubules, and vimentin filaments) was accounted for the change in cell morphology of heat-treated CGTH W-2 cells. Integrin and FAK were undetectable after 3 h at 43°C, while vinculin and actin could still be detected, though at a weaker staining intensity, suggesting that integrin and FAK are early targets in the hyperthermic response. Dephosphorylation of FA components was also noted, as shown by anti-phosphotyrosine staining. It is not clear which protein within the FA was dephosphorylated by hyperthermia, although FAK appears to be a candidate. Loss of integrins on the cell surface might result in dephosphorylation of FAK, since activation of the integrin β subunit induces FAK phosphorylation [Guan et al., 1991; Kornberg et al., 1991, 1992]. Tyrosine phosphorylation of FAK is required for the assembly of FA and actin stress fibers [Burridge et al., 1992; Defilippi et al., 1995]. The loss of FAK phosphorylation may be partially responsible for the disassembly of FA and stress fibers.

It is interesting to note that phosphotyrosine staining were lost from the FA after treatment at 43°C for 3 h. However, after 3 h of heat treatment, the vinculin-positive peripheral FA were still intact, suggesting that dephosphorylation of FA components occurred prior to FA disassembly. This observation agrees well with the finding of Burridge et al. [1992] that the decreased phosphotyrosine staining seen in FAs is associated with decreased FA assembly. The peripheral FA are newly-synthesized when cells

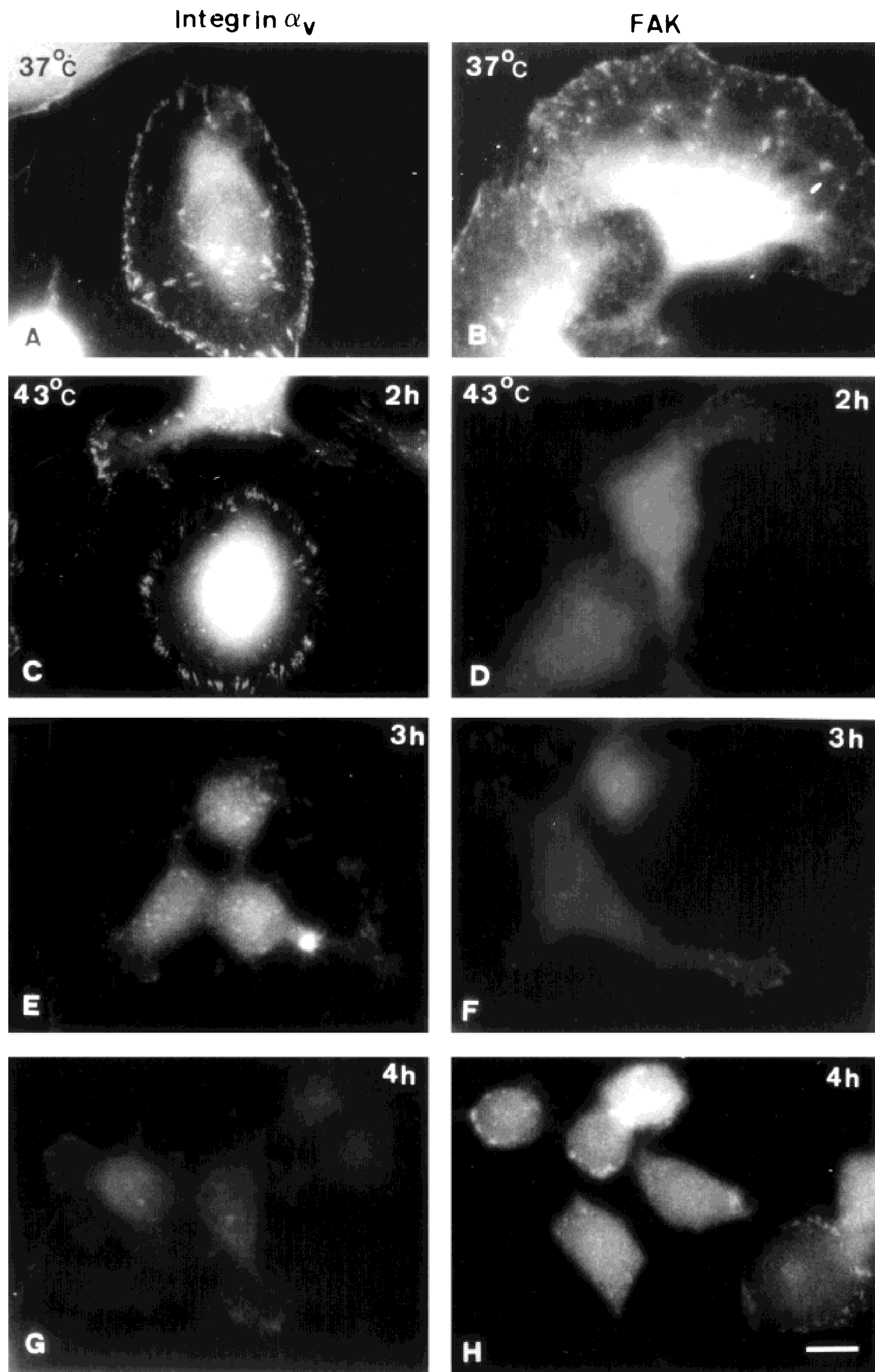

Fig. 7. Effect of heat shock on distribution of integrin αv and focal adhesion kinase in CGTH W-2 cells. **A,C,E,G:** Integrin αv. **B,D,F,H:** Focal adhesion kinase. A,B: Control. C–H: 43°C for 2, 3, or 4 h, respectively. Staining for integrin αv (A) and focal adhesion kinase (B) is located at focal adhesions in control cells. After 2–4 h at 43°C, staining for focal adhesion kinase becomes weak (D,F,H), while integrin staining persists at 2 h (C) of treatment, but fades at 3 (E) and 4 h (G) of treatment. Scale bar = 10 μm.

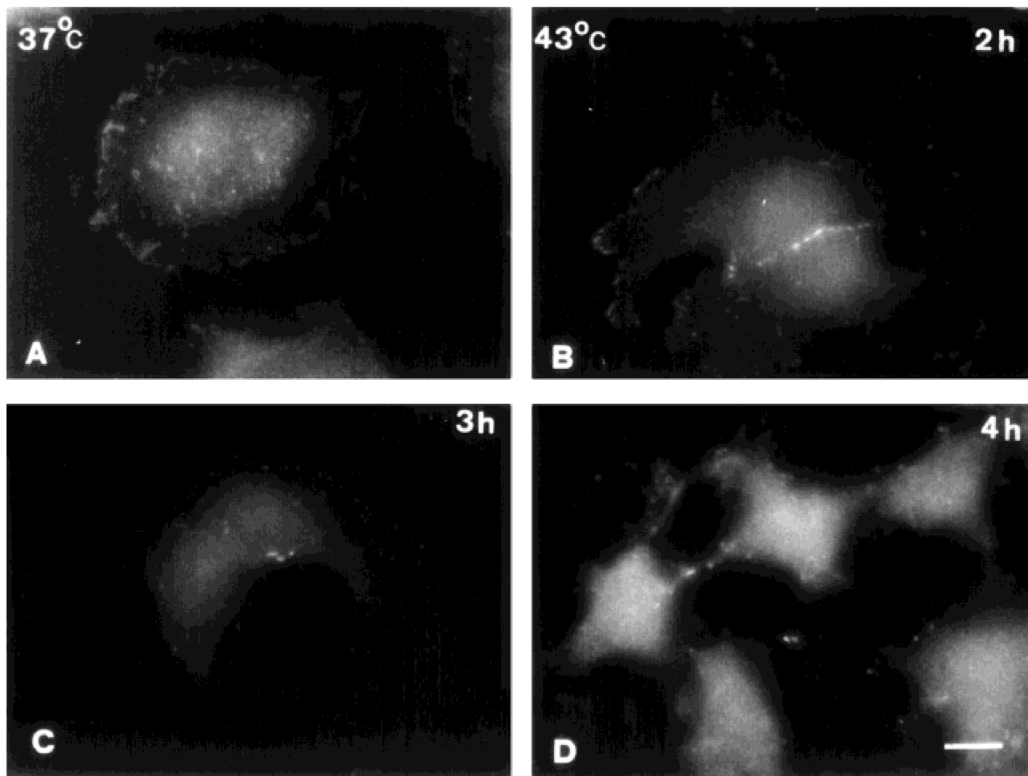

Fig. 8. Effect of heat shock on distribution of phosphotyrosine. Phosphotyrosine staining is located in focal adhesions in control cells (**A**). After heat shock at 43°C for 2 h (**B**) and 3 h (**C**), staining of the central focal adhesion is lost, while that of the peripheral FA is weak. Only weak, diffuse staining is seen after 4 h of treatment (**D**). Scale bar = 10 μm.

are plated on extracellular matrix, the central FA appearing later [DePasquale and Izzard, 1987]. It is plausible that different mechanisms for disassembly in response to hyperthermia may exist in the central and peripheral FAs of CGTH W-2 cells. In the present study, the actin cytoskeleton was completely depolymerized after 5 h at 43°C and this was accompanied by a decreased vinculin staining. Since the barbed (growing) ends of actin filaments are attached to the FA [Kreis et al., 1982; Wang, 1984] and FAs are actin nucleation sites [Small, 1988], the actin cytoskeleton is not maintained once FAs are disassembled, as was the case in this study.

Normal human thyroid tissue expresses α3β4 [Serini et al., 1996]. Integrin α3, α6, and αvβ3 were measured, however, only integrin αvβ3 we detected in CGTHW-2 cells, the vitronectin receptor. The new expression of this particular type of integrin may be due to culture conditions, since integrin αv can be up-regulated in in vitro cultured adherent cell lines [Horton, 1990]. This type of integrin appeares to be more sensitive to heat shock; for example, cell surface levels of integrin αvβ3 are decreased dramatically by heat shock in A549 human lung tumor cells [Majda et al., 1994]. Since integrin is also involved in the adhesion-induced phosphorylation of mitogen-activated protein kinase [Chen et al., 1994], which regulates gene expression by activating transcription factors [Hill and Treisman, 1995], loss of integrin from the cell surface after hyperthermia may block this signaling pathway, together with integrin-mediated actin cytoskeleton assembly.

This study clearly demonstrates that, in addition to affecting certain membrane proteins involved in intracellular pH regulation, cellular Ca^{2+}, and lipid-protein interaction [Konings and Ruifrok, 1985], hyperthermia disrupts the cell-extracellular matrix interaction by targeting integrin-FA complexes. Biochemical events, such as phosphorylation of tyrosine-containing proteins within the FA and FA assembly, that are normally triggered by integrin activation were inhibited following heat treatment, resulting in FA disassembly and actin cytoskeleton depolymerization. The distribution pattern of intermediate filaments was also changed from

filamentous to diffuse by an, as yet, unknown mechanism. Hyperthermia also induced microtubule depolymerization, which arrested the cell cycle of the CGTH W-2 cells, as reported for other cell types [Coss et al., 1982]. This study provides the morphological basis for the effects of hyperthermic treatment seen with CGTH W-2 cells.

ACKNOWLEDGMENT

We Thank Dr. Thomas Barkas for his critical reading and correcting on this paper.

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