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Effect of Tranexamic Acid Incorporated in Fibrin Sealant Clots on the Cell Behavior of Neuronal and Nonneuronal Cells

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Fibrin sealants are commonly used for hemostasis following surgery on various types of tissues. Aprotinin, an effective fibrinolysis inhibitor, is one of the components in some fibrin sealant products currently available. Tranexamic acid (tAMCHA) is another fibrinolysis inhibitor and is used as an alternative to aprotinin. Recent studies on fibrin sealant products containing tAMCHA indicate that it may be responsible for various adverse reactions when used in neurological applications. To determine a possible mechanism for such adverse reactions, we examined the effect of tAMCHA on the behavior of neuronal and nonneuronal cells using in vitro assays. The data indicate that different concentrations of tAMCHA incorporated in fibrin clots had no effect on the initial cell adhesion of either proliferative cells (glial cells and fibroblasts) or nonproliferative cells (neuronal cells) to the fibrin clots. Moreover, a high concentration of tAMCHA (300–450 mM) incorporated in the fibrin clots increased glial and fibroblast proliferation on fibrin clots. However, because tAMCHA is known to leach out of the fibrin clots, we have also examined the effect of solubilized tAMCHA in a growth medium on cells seeded on matrix-coated surfaces. A high concentration (300–450 mM) of tAMCHA detached all cell types from matrix-coated dishes. Our model suggests that tAMCHA in fibrin clots has no adverse effect on cells bound to the fibrin clots; however, tAMCHA leaching out from the fibrin clots reduces adhesion of adjacent cells bound to their natural extracellular matrix. Thus, a high concentration of tAMCHA should not be used as a fibrinolysis inhibitor in fibrin sealant products, especially in neurosurgery.

Key words: neurons; fibrin sealant; adhesion; proliferation

Fibrin sealant products are used to achieve hemostasis and tissue sealing in various surgical procedures, including neuronal tissue applications (Wolner, 1982; Schlag and Redl, 1986; Colman et al., 1987; Narakas, 1988; Ishimura et al., 1991). Fibrin sealant consists of two main components isolated from human plasma, a fibrinogen-enriched complex and thrombin, which form a clot when mixed. Clot formation occurs when fibrinogen, a soluble plasma protein, is converted into insoluble fibrin monomers, via proteolytic cleavage by thrombin. These monomers aggregate into fibrils to form a three-dimensional biopolymer clot that is, ultimately, degraded via proteolysis by plasmin, and the degradation products are then resorbed by phagocytosis (Martinowitz and Saltz, 1996).

In an attempt to increase the longevity and integrity of the fibrin clot, manufacturers of commercially available fibrin sealant products incorporate a fibrinolysis inhibitor into the product. Aprotinin is one of the more common fibrinolysis inhibitors used in fibrin sealant products, such as Tisseel. Tranexamic acid [4-(aminmethyl)cyclohexane carboxylic acid (tAMCHA)] is another fibrinolysis inhibitor used as an alternative to aprotinin. tAMCHA is a lysine analog that blocks reversibly the lysine binding sites on plasmin(-ogen), preventing its binding to fibrin (Hoylaerts et al., 1981; Longstaff, 1994; Dunn and Gao, 1999). In the presence of tranexamic acid, neither plasminogen nor the activated proteolytic enzyme plasmin is able to bind to the lysine residues in fibrin, and thus clot degradation is inhibited (Dunn and Gao, 1999). Moreover, tranexamic acid alone has been used for many years to control bleeding and reduce overall blood loss in various types of surgical procedures, including cardiovascular and oral surgery (Ramstrom and Blomback, 1975; Vermeulen et al., 1984; Dunn and Goa, 1999), with only minimal side effects (Ramstrom and Blomback, 1975). However, several studies have reported that tAMCHA solution (Pelligrini et al., 1982) or tAMCHA incorporated in fibrin clots (Schlag et al., 2000, 2002) induces generalized epilepsy when applied topically to both hemispheres of the cerebral cortex at a low concentration of 1–3 mM. The mechanism is not completely understood, but it appears that a direct interaction of tAMCHA with the cortical surface is required to induce epileptic activity (Pelligrini et al., 1982).

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Although the effect of tAMCHA on nervous tissue has not been extensively studied, it is becoming clear that tAMCHA may not be appropriate for use as a fibrinolysis inhibitor in fibrin sealant products.

The purpose of this study is to examine the effects of varying concentrations of tAMCHA on the behavior of human-derived cells. We examined the effect of tAMCHA incorporated into fibrin clots or solubilized in a growth medium on cell adhesion, cell proliferation, and cell viability using neuronal and nonneuronal human-derived cells. The data support the model that, although tAMCHA incorporated in fibrin clots has no deleterious effects on cells proximal to the fibrin clots, tAMCHA leaching out of the fibrin clots detaches cells that are attached to their natural extracellular matrix. These results suggest that tAMCHA alone or incorporated in fibrin clots should not be used in surgical applications.

MATERIALS AND METHODS

Cell Lines

Glioblastoma cell line (ATCC, Rockville, MD) was maintained in RPMI 1640 media (Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL). The medium also contained the following substrates: adenine (10 mg/liter), adenosine triphosphate (1 mg/liter), and L-cysteine (100 mg/liter); these chemicals were purchased from Sigma (St. Louis, MO). The medium also contained hypoxanthine (15 mg/liter), L-proline (70 mg/liter), sodium pyruvate (100 mg/liter), thymidine, (1 mg/liter), isoleucine (50 mg/liter), and Hepes (5,958 mg/liter); these chemicals were purchased from Gibco BRL. Cells were passaged by rinsing with Hepes-buffered saline solution (HBSS; Cloneticss-BioWhittaker, Walkersville, MD) and trypsinizing with trypsin-EDTA (Clonetics-BioWhittaker), then counted by mixing 100 μl of the cells with 0.4% Tryphan blue (Sigma) and replating in T-cell culture flasks (Sarstedt, Newton, NC).

Neural human normal progenitor (NHNP; Cloneticss-BioWhittaker) cells were not propagated but were used directly in the experiments. NHNP cells were removed from cryopreservation by thawing rapidly in a water bath at 37°C, pipetted slowly several times to resuspend, and delivered equally into two T-75 flasks containing neural progenitor maintenance medium (NPMM; Clonetics-BioWhittaker). The flasks containing cells were incubated in a 5% CO_2 incubator at 37°C for 24 hr, allowing them to recover from cryopreservation prior to use in the adhesion and proliferation assays.

Normal human dermal fibroblasts (Clonetics-BioWhittaker) were propagated in a fibroblast growth medium (FGM; Cloneticss-BioWhittaker) containing fetal bovine serum (FBS; Cloneticss-BioWhittaker). All cell lines were maintained under standard cell culture conditions at 37°C in a 5% CO_2 incubator (Precision Scientific, Chicago, IL).

Preparation of Clots and Matrix-Coated Dishes

The sealer protein (fibrinogen-enriched complex) and the thrombin, components of Tisseel (Baxter AG, Vienna, Austria), were reconstituted as described in the product insert. Briefly, the sealer protein component was reconstituted in aprotinin solution, and the thrombin component was reconstituted in 40 mM $CaCl_2$. The aprotinin solution in some cases was replaced with a solution of tAMCHA (Sigma) dissolved in dH_2O and was filtered to obtain sterile solutions. The initial tAMCHA concentrations used to prepare the sealer protein solutions were 62.5, 300, 600, or 900 mM (Table I). The final clots were prepared as follows: 150 μl of the sealer protein solution was added evenly to the wells of a 24-well culture plate (Becton Dickinson, Franklin Lakes, NY). Then, 150 μl of the thrombin solution was added to the same wells and quickly mixed with the sealer protein solution by tilting the plates back and forth. The plates were left at room temperature for 2 hr and then overlayed with either aprotinin in Tris-buffered saline (TBS; Sigma) or the same tAMCHA concentration in TBS that was used to prepare the sealer protein solution; for example, if the final concentration of tAMCHA in the final clot is 150 mM, then the final concentration of tAMCHA in the solution added to the clots is also 150 mM. The plates were then stored at 4°C. Polyethyleneimine (PEI)-coated dishes were prepared by adding 0.5 ml of PEI (Clonetics-BioWhittaker) for at least 3 hr at 37°C and were rinsed three times with sterile water before use.

Cell Adhesion Assay

The fibrin sealant clots, prepared as described above, were first rinsed twice with 1 ml of a serum-free medium, and 0.3 ml of the same medium were then added to the clots and the plates read using a fluorescent plate reader (PerSeptive Biosystem, Framingham, MA). This measurement represents a background reading. Cells were harvested, counted using a hemacytometer, rinsed twice with 10 ml of a serum-free RPMI 1640 medium (Gibco BRL), and resuspended in 1 ml of the same medium. The cells were labeled by adding 5 μl of 4 mM BCECF.AM (Molecular Probes, Eugene, OR), followed by incubation at room temperature in the dark for 50 min. The cells were then rinsed twice with a serum-free medium and resuspended in the same medium. Next, approximately 10,000 cells (50,000 cells for NHNP) were added to each well and incubated for 2 hr at 37°C in a 5% CO_2 incubator. The plates were read to determine the fluorescence intensity corresponding to the total number of cells added to the clots. The clots were then rinsed twice with a serum-free medium to remove the nonadherent cells, and 0.3 ml of a serum-free medium were added to the wells. The plates were then read again. This reading corresponded to the number of cells adhered to the clots. After subtraction of the background reading from both the total cell number and the adherent cell readings, the percentage adhesion was calculated as the percentage of adherent cells to the initial total number of cells added to the plates.

The adhesion of glioblastoma to collagen-coated dishes (Becton Dickinson, Bedford, MA), fibroblasts to tissue culture dishes (Becton Dickinson, Frankline Lakes, NY), and NHNP to PEI-coated dishes was performed as described above in the presence of different concentrations of tAMCHA in media. The determination of cell adhesion to each clot formulation or to the coated dishes was repeated a minimum of 10 times each. Standard deviations were calculated for at least $n = 10$.

Cell Proliferation Assay

Five identical plates were prepared for each clot formulation or matrix-coated dish tested as described for the cell adhesion assay. On the next day, cells were added to four plates, followed by incubation for 2 hr at 37°C in a 5% CO_2 incubator. The plates were rinsed twice to remove the nonadherent cells, and a serum-containing medium was added to three plates. The fourth plate containing cells and the control plate that did not contain cells were labeled with a live/dead working dye solution (10 μM EthD-1 and 5 μM CalceinAM; Molecular Probes) at room temperature in the dark for 50 min. The plates were then read using a fluorescence plate reader. The reading from the control plate without cells represented the background. The reading from the plate containing cells (after subtracting the reading from the background plate) represented the number of cells at the beginning of the experiment. The remaining three plates were incubated at 37°C in a 5% CO_2 incubator, with the growth medium changed daily. The live/dead labeling procedure was repeated daily for 3 days using one of the three remaining plates. The proliferation index, or increase in cell number, was defined as the percentage of fluorescent intensity measured at day 1, 2, or 3 compared with the fluorescent intensity reading of the first day. The cell proliferation for each

TABLE I. tAMCHA Concentrations in Clots or in Solution: Final Concentration in the Clots Is Half the Concentration of tAMCHA Added To Make the Fibrinogen Solution

Clot/ solution	Tisseel	Reconstituted solution (mM)	[tAMCHA] in clot or solution (mM)
Clot 1	+	Aprotinin	0.00
Clot 2	+	62.5 tAMCHA	31.25
Clot 3	+	300 tAMCHA	150
Clot 4	+	600 tAMCHA	300
Clot 5	+	900 tAMCHA	450
Solution 1	−	62.5 tAMCHA	31.25
Solution 2	−	300 tAMCHA	150
Solution 3	−	600 tAMCHA	300
Solution 4	−	900 tAMCHA	450

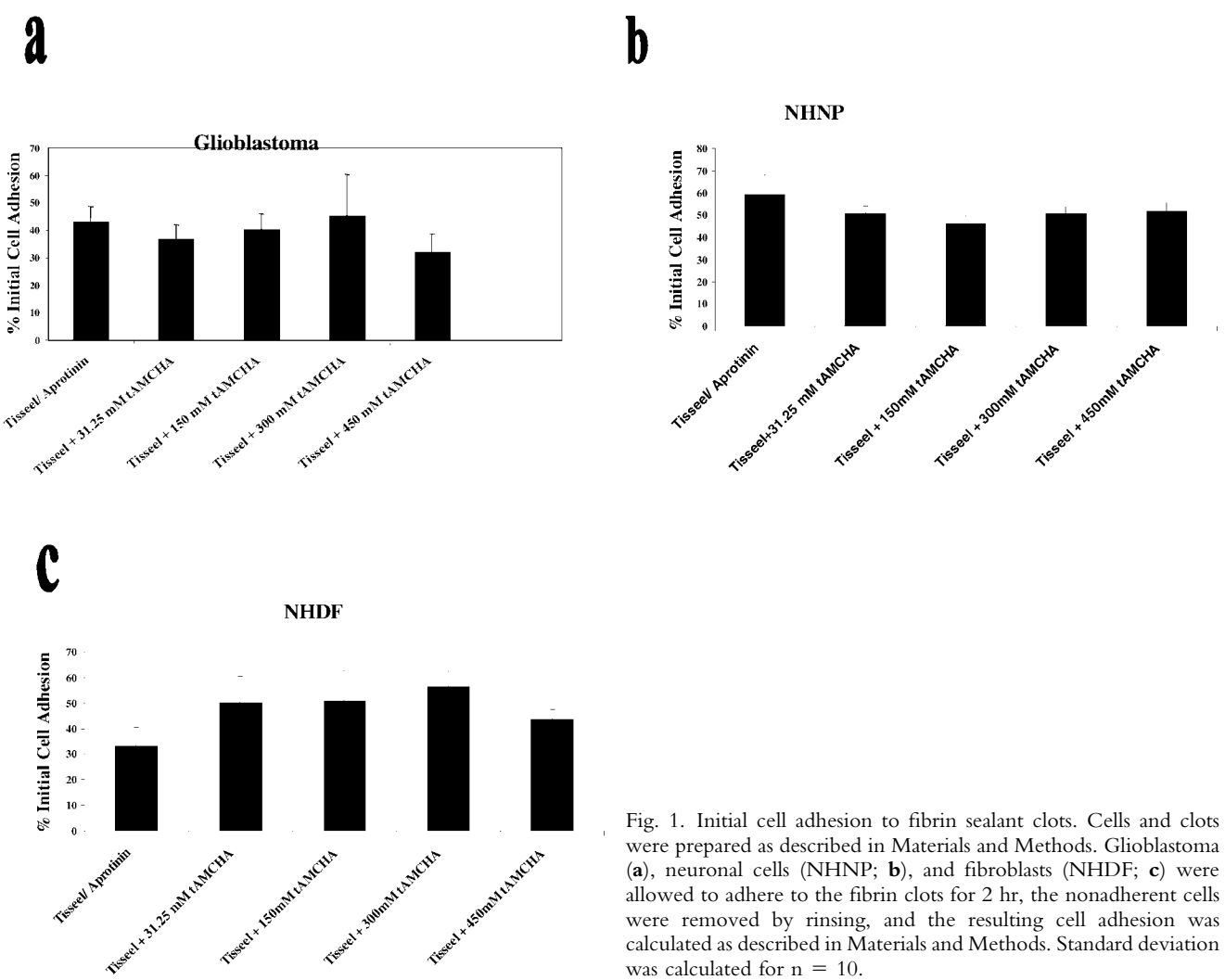


Fig. 1. Initial cell adhesion to fibrin sealant clots. Cells and clots were prepared as described in Materials and Methods. Glioblastoma (**a**), neuronal cells (NHNP; **b**), and fibroblasts (NHDF; **c**) were allowed to adhere to the fibrin clots for 2 hr, the nonadherent cells were removed by rinsing, and the resulting cell adhesion was calculated as described in Materials and Methods. Standard deviation was calculated for n = 10.

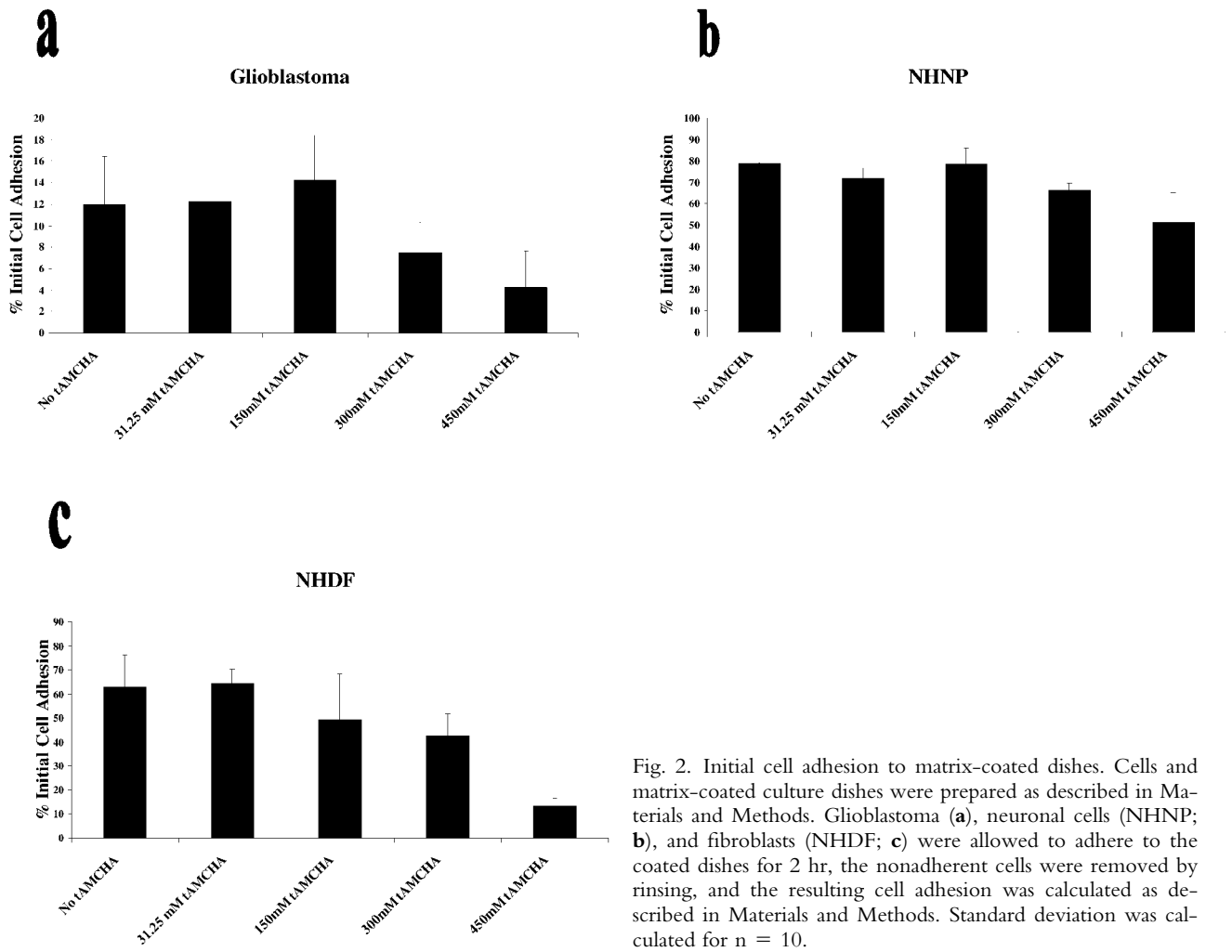


Fig. 2. Initial cell adhesion to matrix-coated dishes. Cells and matrix-coated culture dishes were prepared as described in Materials and Methods. Glioblastoma (**a**), neuronal cells (NHNP; **b**), and fibroblasts (NHDF; **c**) were allowed to adhere to the coated dishes for 2 hr, the nonadherent cells were removed by rinsing, and the resulting cell adhesion was calculated as described in Materials and Methods. Standard deviation was calculated for n = 10.

clot formulation or coated dish was measured in at least 10 sample wells. The standard deviations were calculated for these samples.

Cytotoxicity Assay and Cell Viability

Cells were thawed and maintained as described above. For studies regarding NHNP cells, 0.1 ml of 1× PEI was added to two 96-well and two 24-well culture plates and incubated at room temperature for at least 3 hr or overnight. Cytotoxicity assay was preformed in the 96-well plates. Wells were then rinsed three times with sterile water, and 0.5 ml of a serum-free medium were added to the wells. NHDF or NHNP was collected from flasks as described above, 10,000 cells were added to each well, 500 μl of a serum-containing medium were added to each well, and the plate was placed in a 5% CO_2 incubator at 37°C for 6–7 days. Medium was changed every 2–3 days. After cells had reached a desirable confluence (70–80%), varying concentrations of t-AMCHA (0.0 mM, 31.25 mM, 150 mM, 300 mM, and 450 mM) in a serum-free medium were added to each well. One hundred microliters of lysis buffer/maximum lactate dehydrogenase release (Max LDH; Promega, Madison, WI) were added to some wells as a positive control for the cytotoxicity assay. One hundred microliters of trypsin were added to other wells as a positive control for observing cell morphology during detachment. Plates were incubated at 5% CO_2 incubator at 37°C. At 8 hr, plates were taken out of the incubator and allowed to equilibrate to room temperature for about 20–30 minutes. One hundred microliters of CytoTox-One reagent (Promega) were added to all wells and incubated at room temperature for 10 min. Then, 50 μl of stop solution were added to all wells and shaken for 30 sec. The plate was read using a fluorescent plate reader at an excitation wavelength of 530–570 nm and an emission wavelength of 580–620 nm. Supernatant including detached cells was collected from the 24-well plates, counted, placed into a new 24-well plate with fresh medium, and monitored for several days for cell attachment and spreading, and then pictures were taken using an inverted microscope (Nikon, Melville, NY).

RESULTS

High Concentrations of tAMCHA Inhibited Initial Cell Adhesion to Matrix-Coated Dishes but Had No Effect on the Cell Adhesion to Fibrin Clots

Three human-derived cell lines were used, including the human glioblastoma cell line, which is known to have characteristics similar to those of normal human glia cells

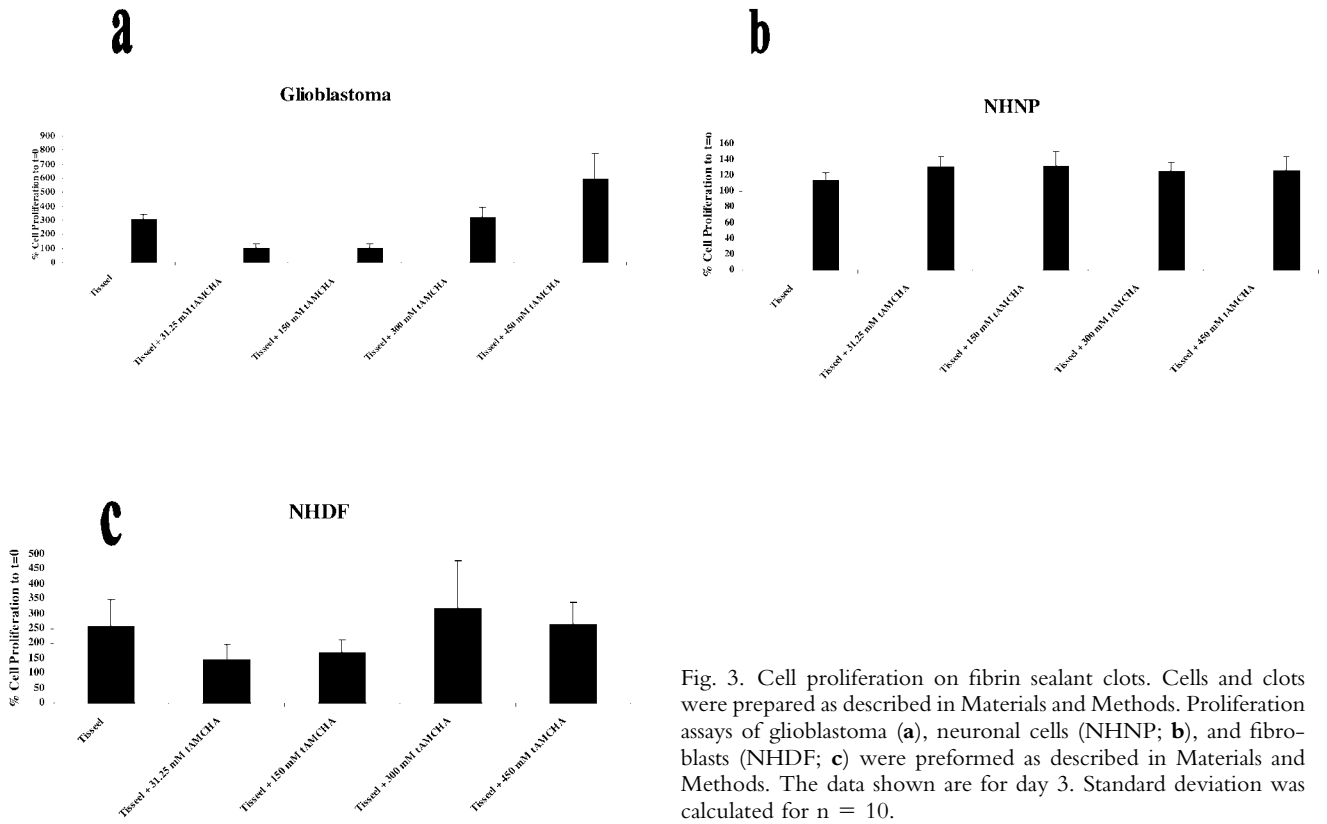


Fig. 3. Cell proliferation on fibrin sealant clots. Cells and clots were prepared as described in Materials and Methods. Proliferation assays of glioblastoma (**a**), neuronal cells (NHNP; **b**), and fibroblasts (NHDF; **c**) were preformed as described in Materials and Methods. The data shown are for day 3. Standard deviation was calculated for n = 10.

(Sundstrom and Nilsson, 1976). Both the human fibroblast and the neuronal cell lines were primary cultures obtained from human tissue samples. The fibrin clots were prepared either from Tisseel reconstituted with aprotinin or with increasing concentrations of tAMCHA (see Table I) by reconstituting the sealer protein with tAMCHA solution instead of aprotinin. The data indicated that tAMCHA incorporated in fibrin clots had no significant effect on the initial cell adhesion to the fibrin clots for the three cell types (Fig. 1).

It was observed that tAMCHA leaches from the fibrin clots, so the effect of tAMCHA in solution on cell behavior was examined. The kinetics of tAMCHA leaching out of the fibrin clots have not yet been fully characterized (Thomas Seelich, Baxter Healthcare Corp., unpublished data). Therefore, various concentrations of tAMCHA (Table I) were added to the growth medium, and the initial cell adhesion to matrix-coated dishes was examined. The matrix to which each cell type is known to adhere the best was used in this assay, i.e., fibronectin for fibroblasts, collagen for glioblastoma, and PEI for the neuronal cell line. A high concentration of tAMCHA (300–450 mM) in the growth medium inhibited the initial cell adhesion of glioblastoma cells (Fig. 2a) and fibroblasts (Fig. 2c) to biological matrix-coated dishes but had no obvious effect on neuronal cell adhesion (Fig. 2b). Taken together, these data suggest that tAMCHA in fibrin clots did not affect the initial adhesion of cells to the fibrin clots. However, tAMCHA leaching out from the fibrin clots into solution could inhibit initial cell adhesion of cells to matrix-coated surfaces, if high concentrations were reached.

Effect of tAMCHA in Clots on Cell Proliferation

Initial cell adhesion to a substrate does not necessarily indicate how cells maintain their adhesion to the substrate over time or how well they will proliferate. Therefore, we examined cell proliferation on fibrin clots containing different concentrations of tAMCHA. For both glial cells (Fig. 3a) and fibroblasts (Fig. 3c), there was a decrease in cell proliferation in the presence of low concentrations of tAMCHA (31.25 and 150 mM) compared with aprotinin-containing clots. On the other hand, there was an increase in cell proliferation in the presence of high concentrations of tAMCHA (300 and 450 mM) compared with aprotinin-containing fibrin clots for the same cell lines.

NHNP is a neuronal cell line, which consists of neuronal spheroids that do not proliferate. Normally, neurons start to migrate out of the spheroids and then start to differentiate and extend neurites. Thus, unlike the effect on fibroblasts and glial cells, the effect of tAMCHA incorporated in the fibrin clots is not on NHNP proliferation but rather on their adhesion and survival. The data indicate that the number of nonproliferative neuronal cells in the spheroids on the fibrin clots was not affected with an increasing concentration of tAMCHA (Fig. 3b). Thus, the

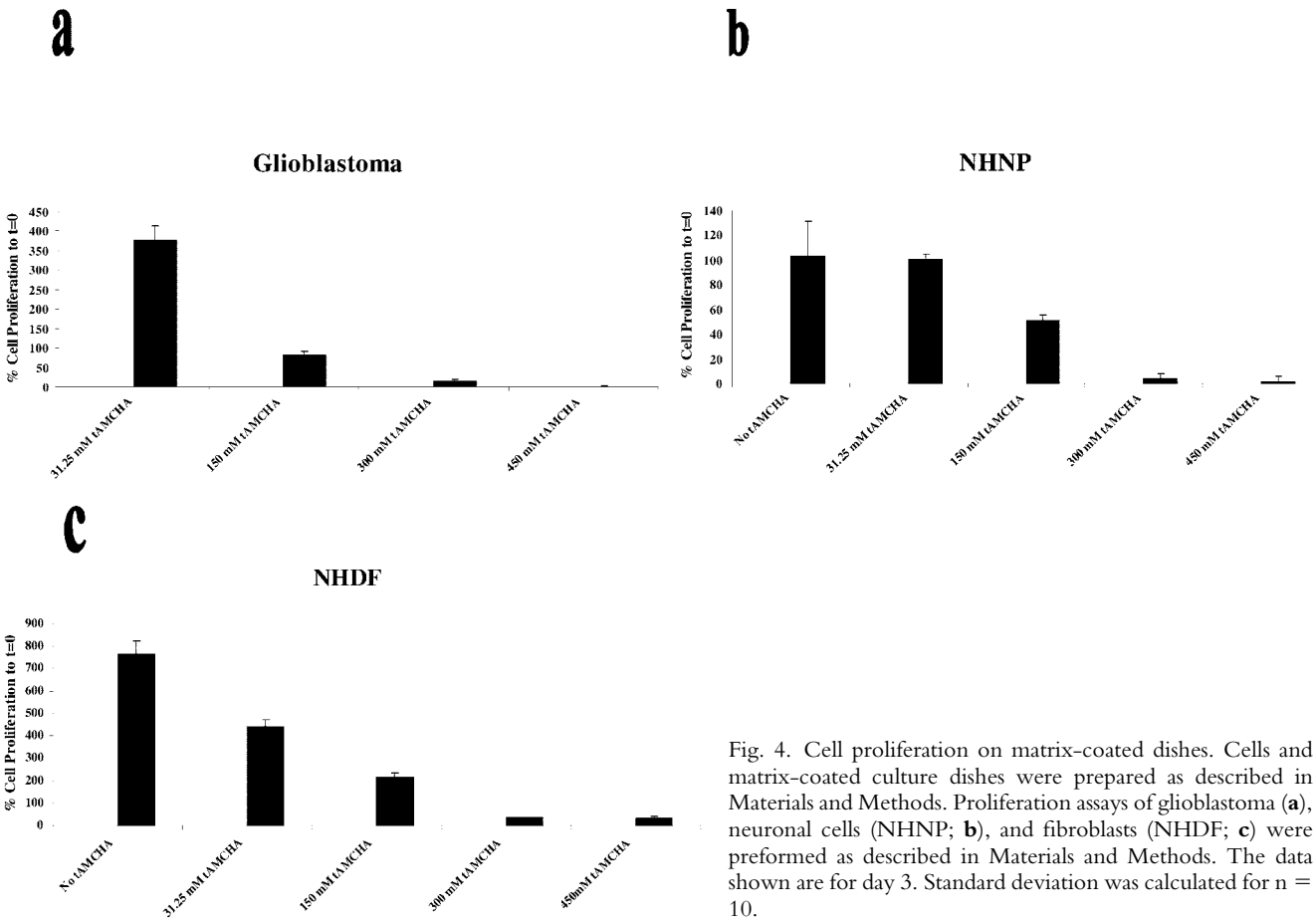


Fig. 4. Cell proliferation on matrix-coated dishes. Cells and matrix-coated culture dishes were prepared as described in Materials and Methods. Proliferation assays of glioblastoma (**a**), neuronal cells (NHNP; **b**), and fibroblasts (NHDF; **c**) were preformed as described in Materials and Methods. The data shown are for day 3. Standard deviation was calculated for n = 10.

concentration of tAMCHA incorporated in the fibrin clots affected only the proliferation of profilerative cells and had no effect on the adhesion of nonprofilerative cells to fibrin clots.

tAMCHA Detached Cells From Matrix-Coated Culture Dishes

tAMCHA leaching out of fibrin clots might affect the behavior of cells adjacent to the clots. Thus, we examined the effect of varying concentrations of tAMCHA in the growth medium on cells seeded on matrix-coated dishes. Cells were maintained in a growth medium containing a known concentration of tAMCHA, and the medium was replenished daily with the same tAMCHA concentration. The data show that, after 3 days, higher concentrations of tAMCHA reduced the number of adhered glioblastoma (Fig. 4a), fibroblasts (Fig. 4c), and neuronal cells (Fig. 4b). Thus, tAMCHA

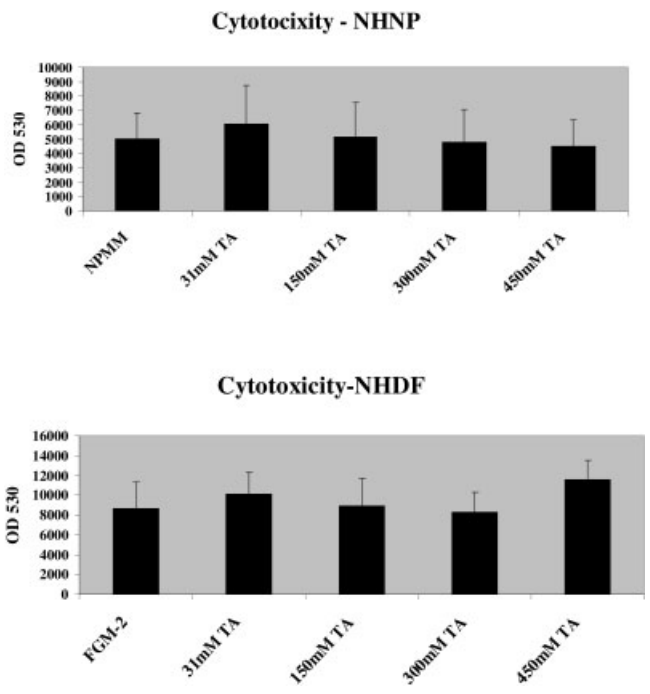


Fig. 5. tAMCHA has no cytotoxic effect on cells. Fibroblasts and neuronal cells were allowed to adhere and spread on coated dishes and then were treated with an increasing concentration of tAMCHA solutions as described in Materials and Methods. **Top:** Fibroblasts. **Bottom:** Neuronal cells.

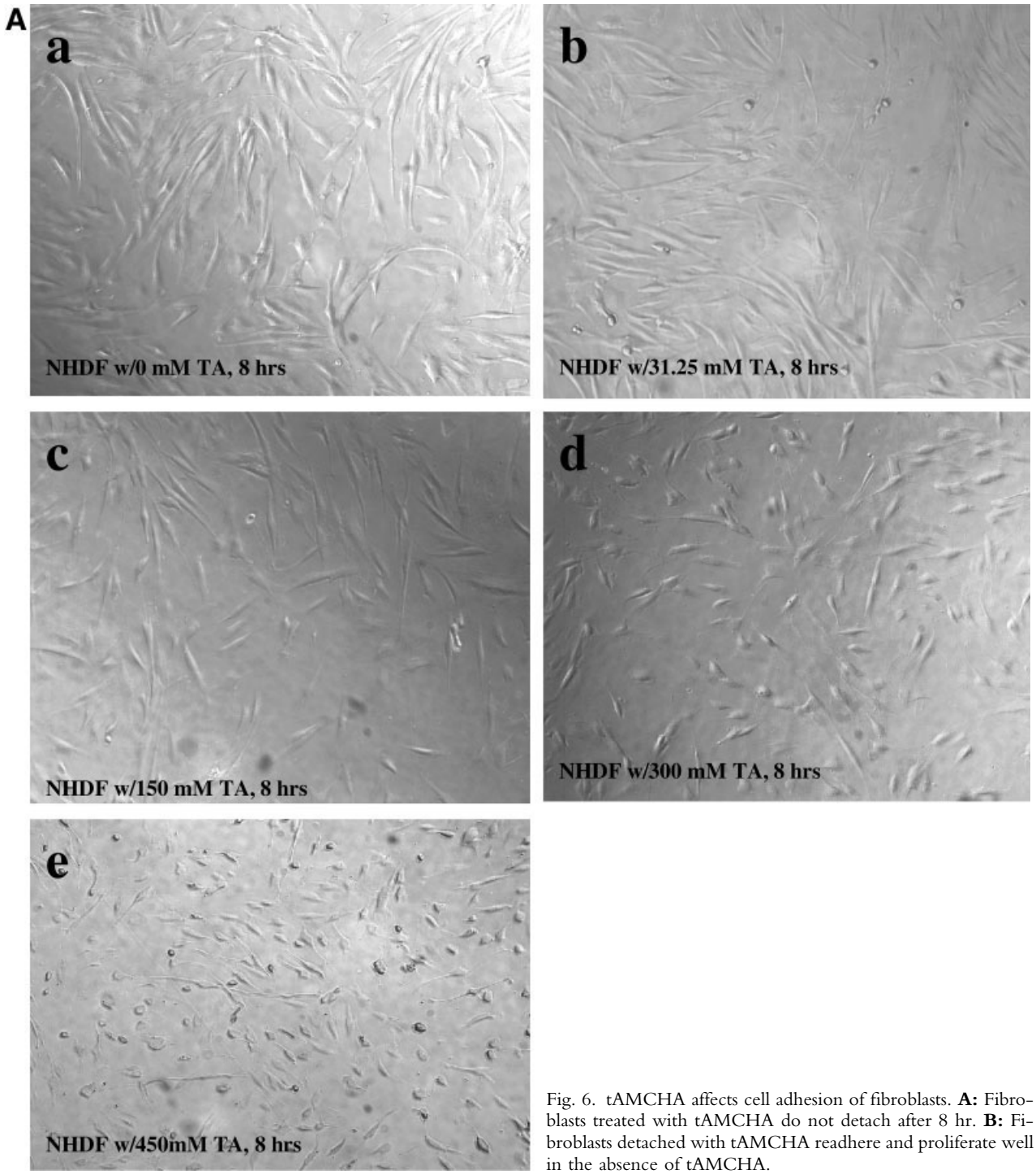


Fig. 6. tAMCHA affects cell adhesion of fibroblasts. **A:** Fibroblasts treated with tAMCHA do not detach after 8 hr. **B:** Fibroblasts detached with tAMCHA readhere and proliferate well in the absence of tAMCHA.

was able to detach cells from matrix-coated dishes, suggesting that tAMCHA leaching out from fibrin clots might affect the adhesion of cells to their matrix.

The detachment of cells from matrix-coated dishes in the presence of tAMCHA may be caused by either cells losing their ability to attach or remaining attached or, possibly, cytotoxicity. To determine the cause of cell detachment, cells were seeded on matrix-coated dishes and allowed to adhere and spread for several days. Eight hours after the addition of tAMCHA, a cytotoxicity test was preformed on the attached and detached cells combined. We found that an increasing concentration of tAMCHA had no toxic effect on the fibroblasts (Fig. 5, top) or neuronal cells (Fig. 5, bottom). Moreover, de-

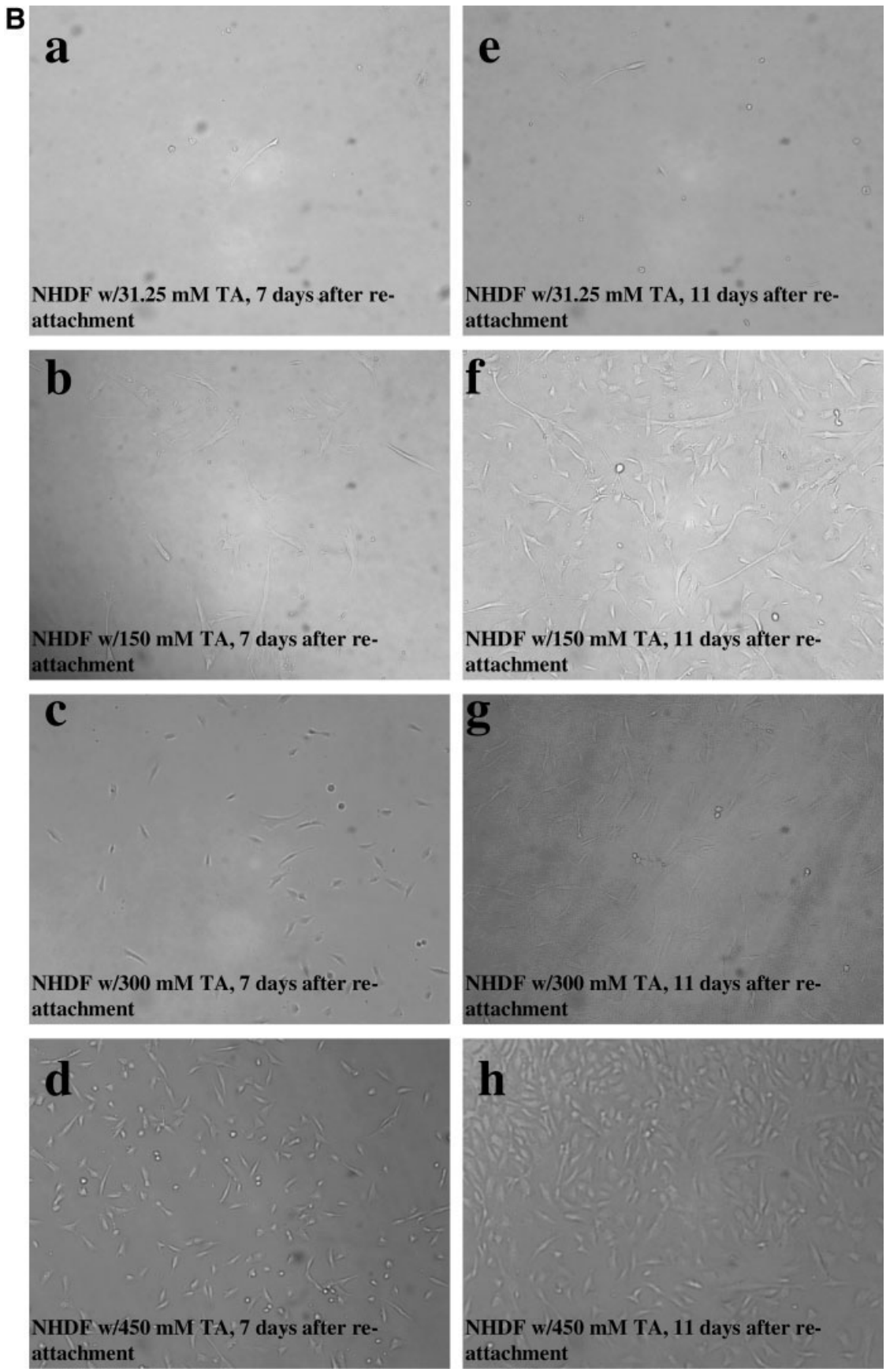


Figure 6. (Continued.)

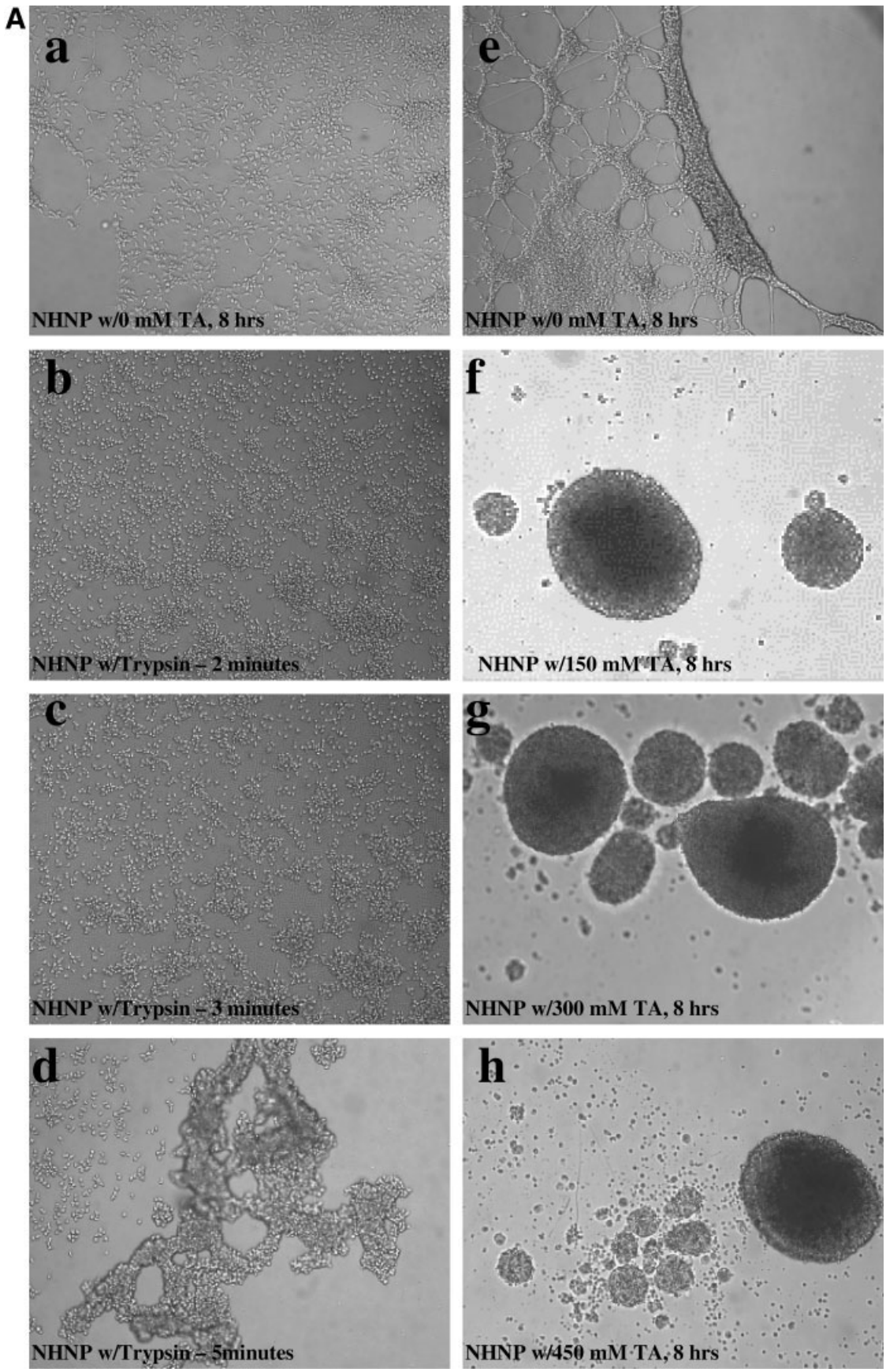
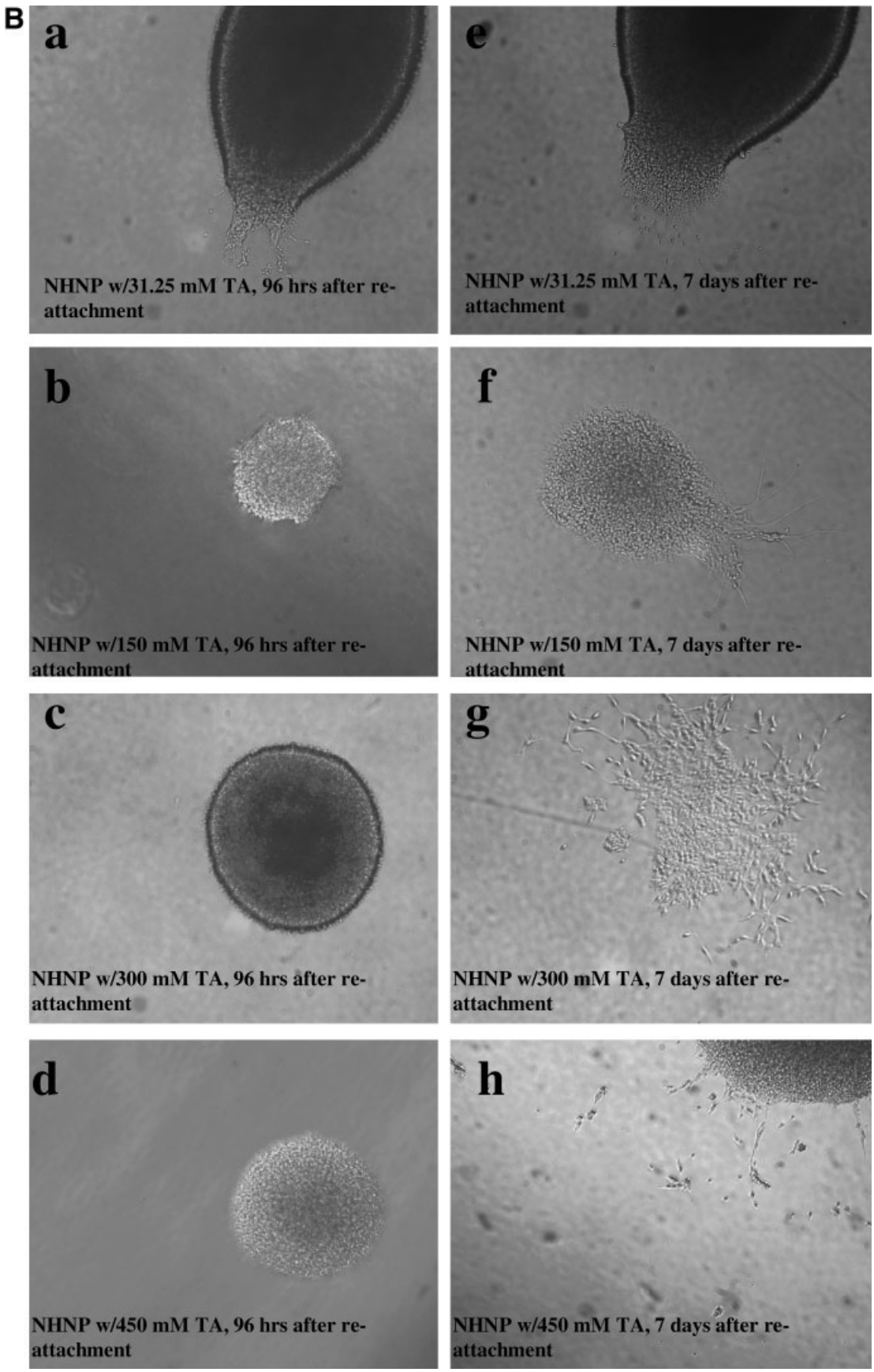


Fig. 7. tAMCHA affects cell adhesion of neuronal cells. **A:** Neuronal cells treated with an increasing concentration of tAMCHA for 8 hr start to retract (e–h), similarly to trypsin-treated neuronal cells (b–d). **B:** Neuronal cells treated with an increasing concentration of tAMCHA for 8 hr were removed from the plates and replaced in a new culture dish in the absence of tAMCHA and allowed to migrate out. **C:** Same as in B for days 7 and 11.



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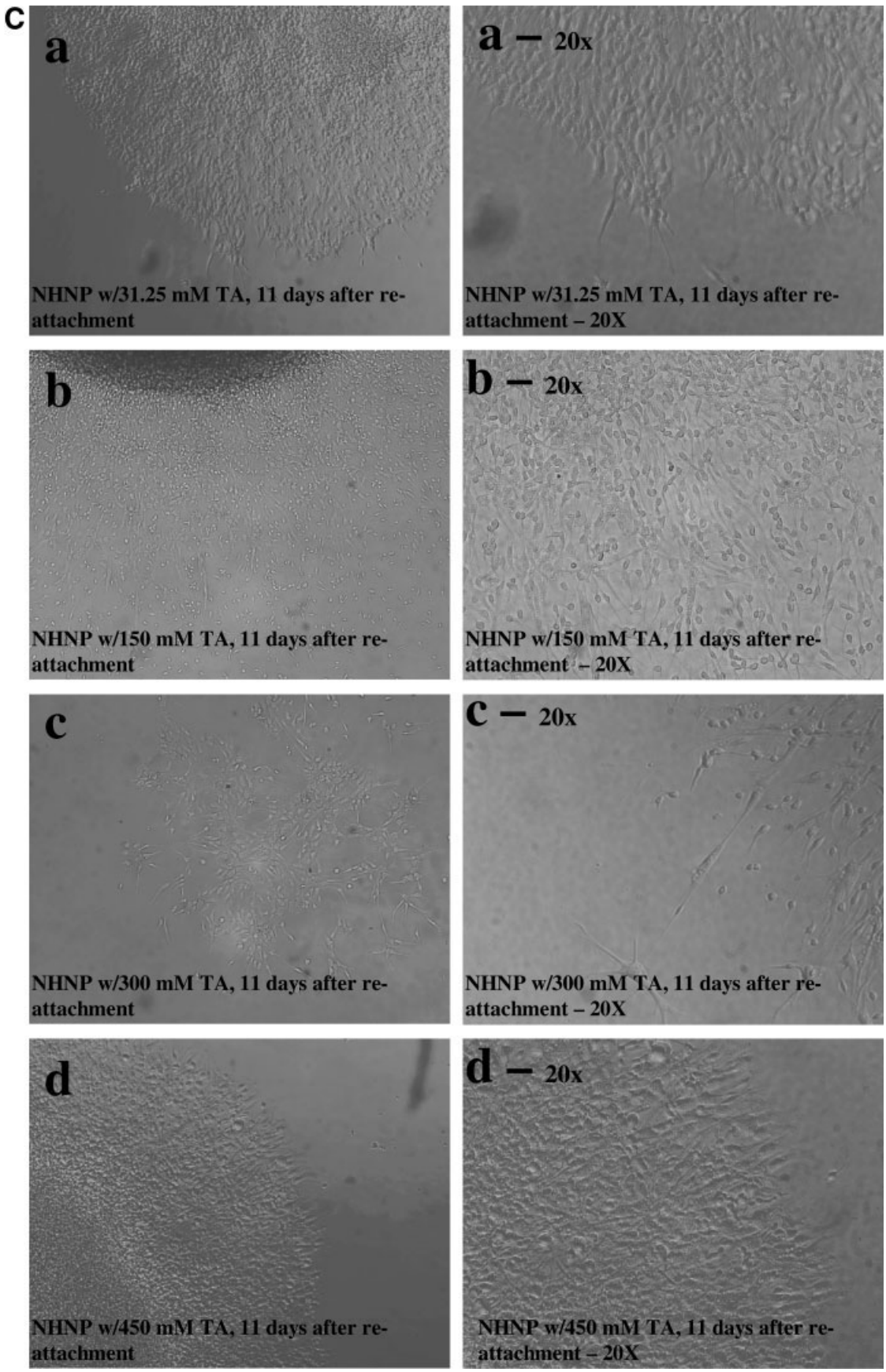

Figure 7. (Continued.)

tached cells were resuspended in a serum-containing medium in the absence of tAMCHA and allowed to adhere and proliferate. Because 8 hr of treatment with tAMCHA did not detach many fibroblasts (Fig. 6A), fibroblasts were instead treated with an increasing concentration of tAMCHA for an additional 16 hr, for a total of 24 hr. We found that fibroblasts that were in suspension readhered to the culture plates and proliferated (Fig. 6B). Because more fibroblasts initially detached with a higher concentration of tAMCHA, more cells were available to readhere, so more cells were seen at day 11 (Fig. 6Bd,h). Similarly, treatment with a high concentration of tAMCHA for 8 hr led to the retraction of neuronal cells back into a spheroid and eventually to the detachment of the spheroid (Fig. 7A). A similar effect was seen with trypsin treatment for 5 min (Fig. 7Ab–d), suggesting that the effect of tAMCHA, similarly to trypsin, is on the adhesion of cells. After removal of tAMCHA, the spheroids reattached, and neuronal cells migrated out of the spheroid again over several days (Fig. 7B,C). The data support a model in which the cell detachment from a coated culture dish in the presence of tAMCHA is due to loss of cell adhesion and not to cell toxicity.

DISCUSSION

Previous studies have shown that, to survive, proliferate, and achieve an optimal cellular response, adherent cells must first adhere to an extracellular matrix protein, such as laminin, collagen, fibronectin, or fibrin, via integrin receptors (Hynes, 1987; Juliano and Haskill, 1993; Giancotti and Ruoslahti, 1999). Our studies (Cole et al., 2001) have shown that various cell types adhere and proliferate well on fibrin clots. It has been demonstrated that human fibroblasts bind directly to fibrin via interactions between the cells and the integrin alpha(v) beta3 receptor (Gailit et al., 1997).

Some fibrin sealant products, such as Tisseel, contain aprotinin as a fibrinolysis inhibitor, whereas other products, such as Quixil, contain tAMCHA as a fibrinolysis inhibitor. In this study, we examined the effect of tAMCHA on cell behavior in vitro, because it has been reported that tAMCHA alone (Pelligrini et al., 1982) or tAMCHA incorporated in fibrin clots (Schlag et al., 2000, 2002) induces epilepsy in animal models when applied directly to the brain. In the present work, fibrin clots containing tAMCHA did not adversely affect the adhesion of proliferative human cell lines, such as normal human dermal fibroblasts or nonneuronal human glial cells to fibrin clots. However, a high concentration of tAMCHA (300–450 mM) caused an increase in fibroblast and glial proliferation on fibrin clots. These data could be explained based on a model in which a high tAMCHA concentration reduces adhesive contacts between the cells and the fibrin clot, leading to an increase in proliferation, insofar as it has been reported that a weakened adhesion to matrix molecules, through modulation of integrin expression, is required for cell division (Tawil et al., 1995; Danen et al., 1996).

More relevant to the in vivo applications of fibrin sealant products containing tAMCHA is the model in which tAMCHA leaching from fibrin clots could affect the adhesion of adjacent cells to the matrix molecules located in the basement membrane even though these cells are not in direct contact with the fibrin clots. Therefore, we examined the effect of tAMCHA solubilized in a growth medium on initial cell adhesion, maintaining attachment and proliferation on matrix-coated dishes. The data indicated that fibroblast adhesion to matrix-coated dishes was reduced over 20% in the presence of 300 mM tAMCHA solubilized in a growth medium and almost 80% with 450 mM tAMCHA. Moreover, cells that initially adhered and spread on matrix-coated dishes overnight in the absence of tAMCHA started to detach when tAMCHA was added, and, by day 3, very few cells remained attached in the presence of a high concentration of tAMCHA (300–450 mM). Similar data were also obtained with the neuronal cell line. These data suggest that tAMCHA leaching out of fibrin clots might lead to detachment of adjacent cells from matrix-coated dishes, and this effect could also occur in vivo, disturbing cell attachment to basement membrane.

It is known that adherent cells spread and form focal adhesions on matrix molecules (Giancotti and Ruoslahti, 1999). Meredith et al. (1993) demonstrated that the detachment of adherent cells from matrix molecule for an extended period causes cells to undergo cell death. It is also known that cells undergoing cytotoxicity detach from matrix-coated surfaces. Therefore, we were interested in determining the cause of cell detachment from fibrin clots in the presence of tAMCHA: Is it a reduction in cell adhesion, an increase in cell toxicity, or both? To answer this question, we examined the initial cell adhesion to matrix-coated dishes in the presence of tAMCHA and found that fibroblasts adhesion was inhibited. Another approach was to seed fibroblasts on matrix-coated dishes in the absence of tAMCHA and allow them to adhere and spread. After a few days, an increasing concentration of tAMCHA was added to the cells for 8 hr; the remaining adhered cells and detached cells combined were then assayed for cytotoxicity. The data clearly showed that tAMCHA did not have a cytotoxic effect on fibroblasts even after 8 hr. The detached cells were rinsed to remove tAMCHA and replated on a culture plate in the absence of tAMCHA. These cells were able to adhere to the plates and proliferate. This indicates that tAMCHA reduces cell adhesion to matrix-coated dishes, perhaps by altering integrin interaction with the matrix molecule.

Moreover, the adhesion and viability of human derived neuronal cells were determined in the presence of varying concentrations of tAMCHA incorporated in the fibrin clot or solubilized in a growth medium. The NHNP cells used in this study were isolated from developing human brain tissue and are found in small aggregates of cells called *neurospheres* or *spheroids* (Mujtaba et al., 1998). When placed into a supplemented growth medium, the spheroids will eventually settle out of suspension and ad-

here to an adhesive substrate, usually within 24 hr. Neuronal progenitors then begin to migrate away from the spheroid and differentiate after migration of individual cells away from the spheroids. In this study, we demonstrated that the initial adhesion of the spheroids to the fibrin clots was unaffected by increasing tAMCHA concentrations and that numbers of NHNP cells decreased only slightly on fibrin clots with an increasing tAMCHA concentration over a 48-hr period. We have also examined the effect of tAMCHA on the adhesion of NHNP cells seeded on matrix-coated dishes. tAMCHA added to confluent NHNP cells did not induce any cell toxicity after 8 hr; instead, the cells started to retract back into the spheroids. This suggests that tAMCHA is affecting the attachment of the cells directly.

In our model, tAMCHA leaching out from the fibrin clots reduces the adhesion of adjacent neurons and nonneuronal cells to matrix-bound basement membrane, possibly by affecting the cell surface expression of integrins or their phosphorylation. This reduction in cell binding to matrix eventually leads to cell death. Cells detached for a long period will eventually undergo apoptosis, as indicated in previous studies (Meredith et al., 1993), because of the loss of adhesion to a matrix molecule. Another model is that tAMCHA induces hyperexcitability by blocking γ-aminobutyric acid (GABA)-driven inhibition of the CNS (Furtmuller et al., 2002). Either of these two models could be invovled in the tAMCHA effect on cells. Whereas the GABA-driven inhibition of tAMCHA might play a more dominat role within minutes after tAMCHA's application to neuronal cells, the tAMCHA effect on cell adhesion is more prominent a few hours after the addition of tAMCHA on cells. These data indicate that tAMCHA used alone or as a fibrinolysis inhibitor in fibrin sealant products in surgery may have adverse effects on neuronal and nonneuronal tissues.

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