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ORGAN TOXICITY AND MECHANISMS

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# Humic acid extracted from Blackfoot disease-endemic well water induces adipocyte differentiation of C3H10T1/2 fibroblast cells: a possible mechanism leading to atherosclerotic-like plaque in Blackfoot disease

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**Abstract** A unique peripheral vascular disease named "Blackfoot disease" (BFD) is endemic on the southwest coast of Taiwan. Clinically, the signs and symptoms of BFD are similar to those of arteriosclerosis and Buerger disease. Humic acid has been proposed as a causative factor in BFD; however, the relationship between humic acid and atherosclerotic-like plaque associated with BFD remains unclear. In this study, we investigated the effects of humic acid extracted from Blackfoot disease-endemic well water (BFD-HA) on cultured C3H10T1/2 fibroblasts, a murine embryonic cell line. Our present data demonstrate that C3H10T1/2 cells were arrested at the $G_1$ phase and subsequently differentiated to adipocytes after treatment with BFD-HA. The adipocyte differentiation of C3H10T1/2 cells induced by BFD-HA was also accompanied with increased glycosaminoglycan production. These results suggest that a large lipid accumulation of arterial blood vessels in BFD patients may be linked in part to the adipocyte differentiation of vascular fibroblasts induced by BFD-HA.

**Keywords** Adipocyte differentiation · Blackfoot disease · Humic acid

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## Introduction

Blackfoot disease (BFD), an endemic peripheral vascular disease confined to the southwestern coast of Taiwan, results in gangrene of the extremities, especially the feet (Tseng et al. 1961) Clinically, the signs and symptoms of BFD are similar to, although much more severe than those of arteriosclerosis and Buerger disease (Tseng 1989). A pathological study has revealed that 30% of BFD patients had features compatible with thromboangiitis obliterans and 70% showed clinical characteristics of arteriosclerosis obliterans (Yeh and How 1963). Although the aetiology of BFD is still not clearly identified, epidemiological studies disclosed a high concentration of humic acid (approximately 200 ppm) in artesian well water from areas of endemic BFD (Chen et al. 1962; Lu et al. 1980). Humic acid extracted from Blackfoot disease-endemic well water (BFD-HA) has been reported to induce blackening of the tail and feet in mice and rats (Lu and Liu 1986; Lu 1990a).

Humic acid is a group of polymers with high molecular weight derived from the decomposition of organic matter, especially dead plants. It exists abundantly in peat, soil, well water and other sources (Hartenstein 1981) and consists of a mixture of closely related compounds with complex polymeric aromatic structures. Reduction, oxidation, and microbial degradation of humic acid produce resorcinol, o- and m-phthalic acids, and other phenolic and phenolic carboxylic compounds (Burges et al. 1964; Choudhry 1981; Keyser et al. 1976). Chemical and infrared spectroscopic analyses revealed the presence of aromatic rings, phenolic hydroxyl, ketonyl, quinone carbonyl, carboxyl, and alkoxyl groups in humic acid (Stevenson 1985). BFD-HA is stable to heat and acid-base action and contains carboxyl, hydroxyl, and carbonyl as the main functional groups and is often complexed with mixtures of metals such as arsenic, iron, manganese, lead, cadmium, zinc, and nickel (Lu 1990b).

A study was carried out to investigate the causes of death in BFD patients, based on 1,075 BFD patients in

Taiwan from 1958–1974. Among of the causes of death, cardiovascular disease accounted for 18% and this was significantly higher than in non-affected residents in the endemic area (Tseng 1975). Pathological findings included thickening of the tunica intima, excess synthesis of collagenous matrix (fibroblastic initimal thickening) and a large accumulation of lipid in the arterial blood vessels of BFD patients; these characteristics seem to be an atherosclerotic plaque (Yeh et al. 1958). It is now recognized that atherosclerotic plaque is highly associated with fatty streaks in blood vessels (Willeit and Kiechl 2000). However, the lipid profiles including total cholesterol, triglyceride, HDL, LDL, apolipoprotein AI and apolipoprotein B were within normal limits in BFD patients (Tseng et al. 1997).

Previously, we have demonstrated that commercial humic acid obtained from Aldrich (AHA) is a potent peroxisome proliferator in mice (Lu et al. 1994a; Lee et al. 1999). AHA could induce the expression of peroxisome proliferator-activated receptors (PPAR) $\alpha$ and $\gamma$ subtypes in rodent cells. Also, the C3H10T1/2 fibroblast cells could differentiate to adipocytes via the expression of PPAR $\gamma$ and the adipocyte P2 (aP2) genes induced by AHA (Lee et al. 1999). After an intraperitoneal injection of AHA (100 mg/kg body weight), the histological picture of blood vessels in treated rats was similar to that of BFD patients (Lu et al. 1994b). It may be suggested that the adipocyte conversion from fibroblasts accounts for the atherosclerotic-like plaque formation in the arterial wall of BFD patients.

The process of adipocyte differentiation in the in vitro cell model is well established (Gregoire et al. 1998). The initial molecular event occurring during adipocyte differentiation is growth arrest. Drastic changes also occur in cell morphology, cytoskeletal components, and the level of extracellular matrix components secreted. In this study, we report that BFD-HA induces adipocyte differentiation mediated by growth arrest and increased glycosaminoglycan production in C3H10T1/2 cells.

## Materials and methods

### Purification of BFD-HA

Humic acid of high purity was prepared according to Aiken and Mantoura et al. with minor modifications (Aiken et al. 1979; Mantoura et al. 1975). Water was taken from a well located in the BFD endemic area in Tainan Country on the southwest coast of Taiwan. The water was acidified to pH 1 with 1 N HCl, subjected to adsorption chromatography on a XAD-7 column and eluted with 1 N NaOH. The eluted alkaline-well water was further acidified to pH 1 with 1 N HCl overnight. Following centrifugation, the supernatant was removed and the precipitated humic acid was redissolved in 1 N NaOH. Such a procedure of alkaline-acid treatment was repeated twice. After the third round of alkaline dissolution, the extracted water was evaporated to a dry powder. The IR spectra of purified humic acid showed the presence of aliphatic C-H, aromatic C = C, C = O of carboxyl groups, C-O and OH groups (Lu et al. 1988). The stock of purified humic acid was dissolved in culture medium (5 mg/ml), the pH was adjusted to 7.4, and then it was filtered through a 0.45μM Millipore filter.

### Cell culture

The C3H10T1/2 (clone 8) fibroblast cell line was obtained from the American Type Culture Collection (Manassas, Va.). The cells were grown in Eagle's basal medium (BME) supplemented with 10% heat-inactivated fetal bovine serum, 50 μg/ml streptomycin, and 50 U/ml penicillin (Gibco, Grand Island, N.Y.). The medium was changed every 3 days and the cells were passaged just before confluence.

### Cell growth assay

C3H10T1/2 cells were plated in duplicate in 100 mm dishes for 18–24 h and the cells were then treated with various concentrations of BFD-HA for 1 to 9 days. Thereafter, trypan blue dye-excluding cells were counted in a haemocytometer.

### Cell cycle analysis

C3H10T1/2 cells were seeded into a 100 mm dish for 18–24 h and the cells were then treated with BFD-HA. The cells that had been treated with BFD-HA were harvested at different points during the time course, washed with ice-cold PBS, resuspended in 1 ml PBS, and fixed in 3 ml of 100% ethanol at –20°C overnight. The cell pellets were collected by centrifugation, resuspended in 0.5 ml of hypotonic buffer (0.5% Triton X-100 in PBS, pH 7.4, and 0.05% RNase), and incubated at 37°C for 30 min. Then 0.5 ml of propidium iodide (PI) solution (50 μg/ml) was added and this mixture was allowed to stand on ice for 30 min. The nuclei were analysed in an FACScan laser flow cytometer (Becton Dickinson, San Jose, Calif.) as previously described (Stokke et al. 1993).

### Adipocyte conversion

C3H10T1/2 cells ectopically expressing adipogenic transcription factors were induced to differentiate into adipocytes as previously described (Taylor et al. 1979). The C3H10T1/2 cells from logarithmically growing stock cultures were seeded into a 100 mm dish. At 2 days post-confluence, the cells were treated with various concentrations of BFD-HA for 24 h. The cells were subsequently fed culture medium until the experiment was terminated. Adipocyte conversion was assessed with lipid accumulation stained with Oil Red O at 2 weeks after treatment according to Kuri-Harcuch et al. 1978. The dishes were examined immediately using a Nikon microscope at ×100 magnification and a focus of adipocytes was defined as 20 or more cells containing lipid droplets within one field of the microscope (ca. 3 $mm^2$).

### Determination of glycosaminoglycan (GAG) production

C3H10T1/2 cells were grown in 6-well plates. At 2 days post-confluence, the cells were treated with various concentrations of BFD-HA. After a 24 h period, the medium containing BFD-HA was discarded, the cells were washed with PBS twice, and fed medium containing 0.4 μCi $^{14}$C-glucosamine (Amersham, Piscataway, N.J.) for an additional 24 h. After labelling, medium was removed and 5 mg/ml of pronase was added for 2 h. The cells were then fully detached by a rubber policeman and incubated at 50°C for 3 h. After centrifugation, 1 mg/ml of hyaluronic acid and chondrointin sulphate (Sigma, St. Louis, Mo.) were added to the supernatant as carrier. The total GAG radioactivity was measured after precipitation with 0.67% cetylpyridinium chloride (Sigma) and collected on Watman GF/F glass fibre filters. Filters were dried at 45°C overnight and a 5 ml scintillation cocktail (ICN, Costa Mesa, Calif.) was added for scintillation counting (Beckman Instruments, Fullerton, Calif.) (Piepkorn et al. 1990; Obunike et al. 2000).

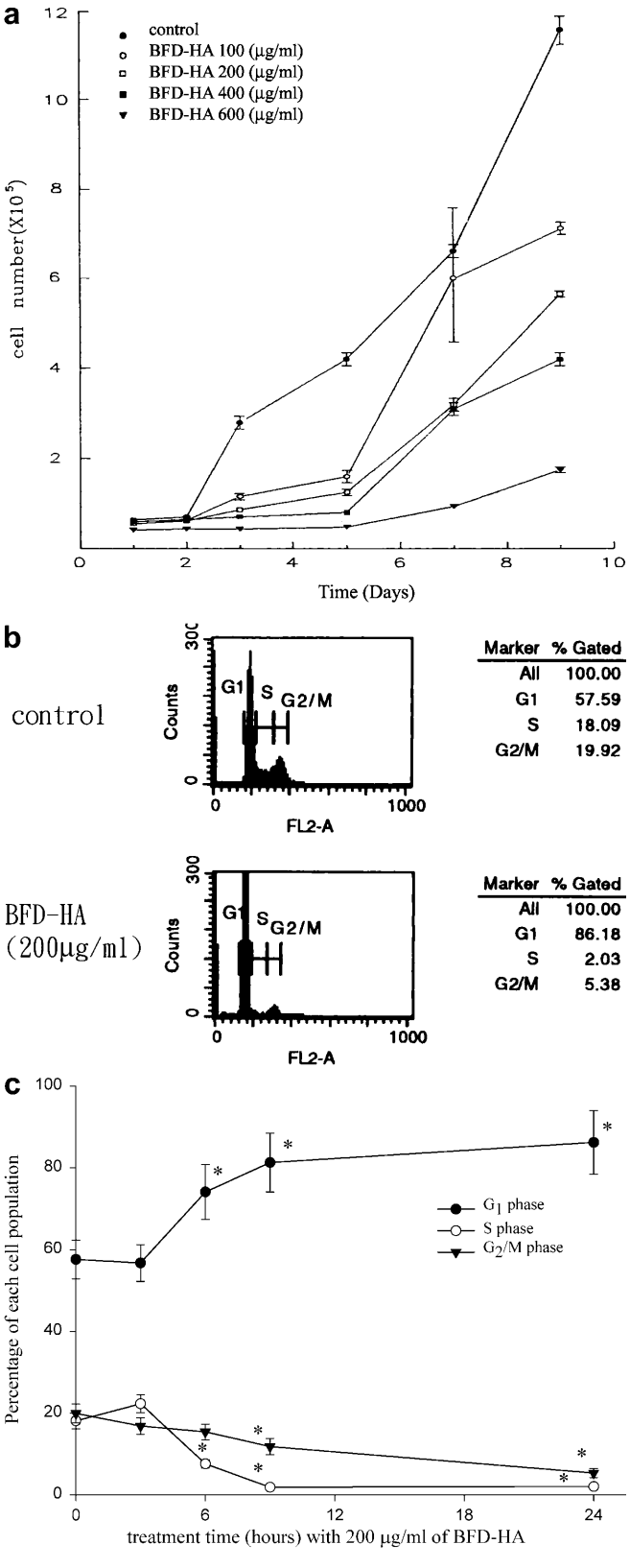


**Fig. 1** Effects of BFD-HA on the growth and cell cycle distribution of C3H10T1/2 cells. (**a**) The cells were treated with various concentrations of BFD-HA for 1 to 9 days. The cell number was counted by trypan blue dye exclusion assay. Each time point is the mean ± SD of two dishes. (**b**, **c**) The cells were treated with 200 μg/ml of BFD-HA. At the indicated times, cells were harvested for flow cytometry. The PI fluorescence profiles of control and BFD-HA treated-groups after 24 h exposure are shown in (**b**). The $G_1$, S and $G_2$/M phases of control and BFD-HA treated groups at various time point are shown in (**c**). Data are presented as means of three independent experiments. $*P < 0.05$ compared to each control

### Statistical analysis

The data were analysed by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test. Differences were considered statistically significant at a value of $P < 0.05$.

## Results

### Effects of BFD-HA on growth of C3H10T1/2 cells and cell cycle progression

Previous studies have shown that humic acid inhibits the growth of many cell lines (Lee et al. 1999; Hofmanová et al. 1999; Chen et al. 2001). In this study, C3H10T1/2 cells were exposed to 100–600 μg/ml of BFD-HA and the cell proliferation was assayed. Exponentially growing C3H10T1/2 cultures rapidly underwent growth inhibition with the addition of BFD-HA, as evidenced by the decrease in cell proliferation over the experimental period (Fig.1a). This inhibitory effect of BFD-HA on cell growth was in a dose- and time-dependent manner. Trypan blue dye exclusion assay indicated marginal toxicity under these conditions (>95% viability even after 600 μg/ml of BFD-HA treatment for 9 days), which suggests that the lower cell count values (Fig.1a) were primarily due to inhibited cell growth rather than cell death.

To clarify whether or not the lower cell count values were due to the cells undergoing apoptosis after application of BFD-HA, we harvested the medium and attached cells for PI staining followed by a flow cytometry assay. There was no sub-G1 DNA content after 600 μg/ml of HA of treatment at each time point (data not shown). Therefore, C3H10T1/2 cells were treated with BFD-HA for 3, 6, 9 or 24 h and then fixed and the cell cycle distribution detected using PI staining in a flow cytometry assay. As shown in Fig.1b, the percentage of cells at the $G_1$ phase (2n DNA content) was increased in the 200 μg/ml of BFD-HA-treated group. Fig.1c shows the results of the $G_1$, S and $G_2$/M phases at each time point (3, 6, 9 and 24 h of exposure) after BFD-HA treatment in three separate experiments. After incubation with 200 μg/ml BFD-HA for more than 9 h, about 80% of cells were arrested in the $G_1$ phase.

## BFD-HA promotes differentiation of C3H10T1/2 cells to adipocytes

To examine whether BFD-HA promoted lipid accumulation in fibroblasts, C3H10T1/2 cells were cultured after 2 days confluence in culture medium supplemented with 200 μg/ml BFD-HA. After 24 h exposure to BFD-HA, the cells were washed twice with PBS and the medium refreshed every 3 days. After an additional 2 weeks of incubation, visual examination of these cultures revealed that the cells accumulated prominent lipid droplets in the BFD-HA treated cells (Fig.2b). As expected, the control cells continued to proliferate until confluence and no lipid droplets were detected in these cells (Fig.2a). Three weeks incubation after the 24 h treatment with BFD-HA, the size of the lipid droplets had increased and could be stained by Oil Red O (Fig.2c,2d).

In preadipose cell lines, growth arrest per se appears to be required for adipocyte differentiation (Gregoire et al. 1998). Since BFD-HA could stop cell growth (Fig.1), we investigated the effect of BFD-HA on adipocyte conversion in exponentially growing C3H10T1/2 cultures. The cells were treated with 200 μg/ml BFD-HA for 24 h at 70% confluence. After another 2 weeks of incubation, the cells could not grow to confluence and adipocyte conversion could be subsequently progressed by BFD-HA (Fig.2e). The numbers of adipocyte foci of C3H10T1/2 cells treated with various concentrations of BFD-HA for 24 h is shown in Fig.3a. Apparently, the formation of adipocyte induced by BFD-HA was in a concentration-dependent manner and if the treatment time of BFD-HA was prolonged (1, 2 or 3 days), more adipocyte foci were scored in C3H10T1/2 cell cultures (Fig. 3b).

## The effect of BFD-HA on GAG production in C3H10T1/2 cells

By keeping adipocytes interconnected during differentiation in culture, the extracellular matrix gave rise to fat cell clusters similar to the lobules found in adipose tissue in vivo (Kubo et al. 2000). To examine alterations in GAG production with cell differentiation induced by BFD-HA, C3H10T1/2 cells, either untreated or treated for 24 h with various concentrations of BFD-HA, were

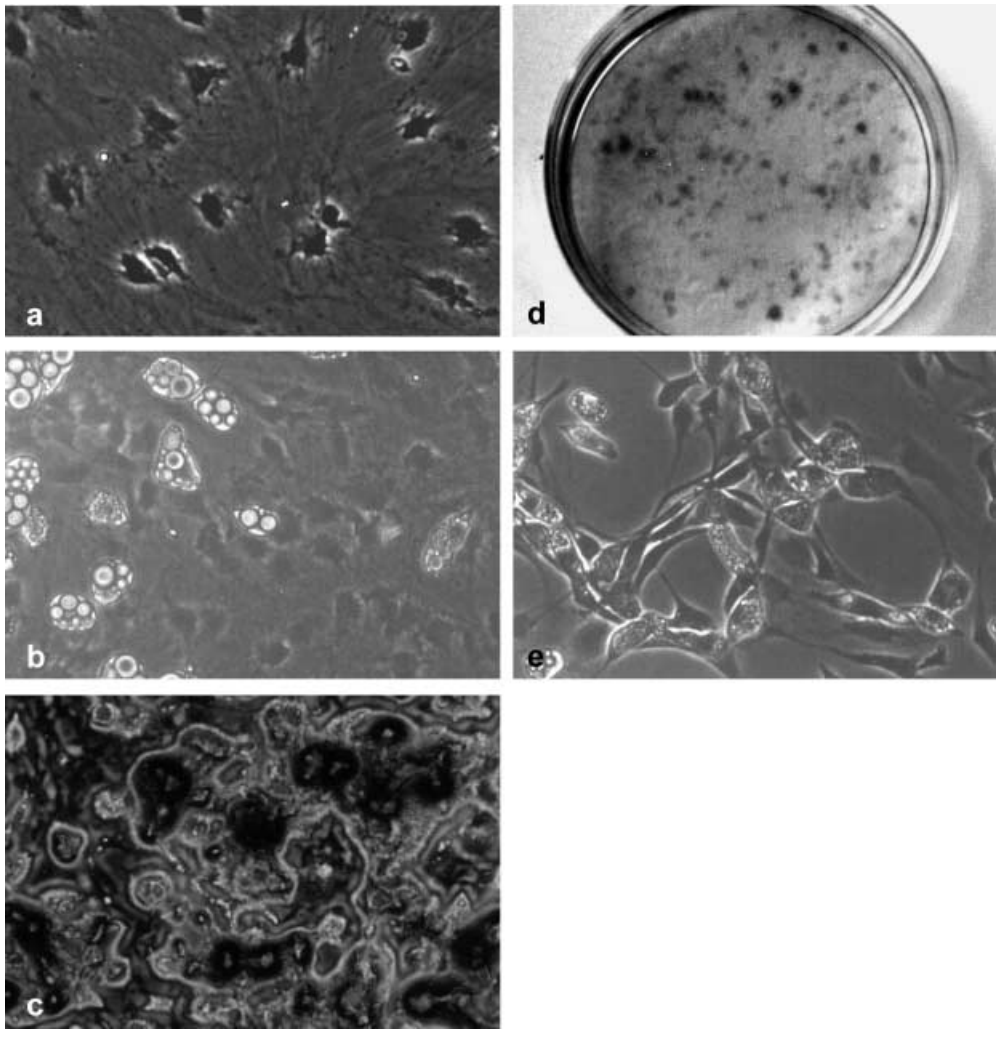


**Fig. 2** BFD-HA promotes differentiation of C3H10T1/2 cells to adipocytes, even in the non-confluent stage. C3H10T1/2 cells were maintained from confluence to day 2 post-confluence and either not treated (**a**) or treated (**b**) with 200 μg/ml of BFD-HA for 24 h. Then the cells remained in culture for 2 weeks. Lipid droplet accumulation is shown after BFD-HA treatment (**b**). At 3 weeks post-treatment, cells were fixed and stained with Oil Red O (**c**, **d**). C3H10T1/2 cells were treated with 200 μg/ml of BFD-HA for 24 h at 70% confluence and then remained in culture for 2 weeks (e). Microscopic views of cells at ×100 magnification are shown in (**a**, **b**, **c**) and (**e**)

radiolabelled for 24 h with $^{14}$C-glucosamine. Incorporation of $^{14}$C was significantly greater in BFD-HA-treated cells than in the untreated cells (Fig.4).

## Discussion

Embryonic cells develop into specialised cell types by a two-step process: determination and differentiation. Determination results in the conversion of embryonic cells into lineages of stem cells. These stem cells proliferate and differentiate and then activate genes that encode the proteins that are functionally appropriate for the differentiated cell type. The C3H10T1/2 cells, derived from C3H mouse embryos, are a well characterised model for adipocyte differentiation (Gregoire et al. 1998). This cell has a fibroblastic morphology and is highly sensitive to post-confluence inhibition of division. It undergoes determination and differentiation into adipocytes in response to appropriate stimulants. In the process of adipocyte differentiation, the cells withdraw from the cell cycle and induce gene expression for fat metabolism, thereby accumulating large cytosolic fat droplets (Reichert and Eick 1999). Additionally, drastic changes occur in cell morphology and cytoskeletal components during adipocyte differentiation. Many of the extracellular matrix components are considered to be necessary for the morphological changes that accompany adipocyte differentiation (Nakajima et al. 1998).

Pathological examination revealed an atherosclerotic-like plaque in the arterial wall of BFD patients (Yeh et al. 1958), including excess synthesis of collagenous matrix and a large accumulation of lipid in the arterial blood vessels. However, the mechanisms leading to the large lipid accumulation in the affected arterial wall remain unclear. Adipose tissue has recently been shown not be simply an energy storage organ, but also a secretory organ, producing a variety of bioactive substances, including leptin, tumour necrosis factor-$\alpha$, plasminogen activator inhibitor type 1 and adiponectin, that may directly contribute to the development of atherosclerosis (Chaldakov 2001). Therefore, the adipocyte differentiation of C3H10T1/2 cells was considered to be a good model to study the atherosclerotic-like plaque in the arterial wall of BFD patients.

In this study, we found that BFD-HA promoted adipocyte differentiation of C3H10T1/2 fibroblast cells. Many adipocyte differentiation studies employ animals and adipose-derived stromal vascular precursor cells as model systems. Growth arrest and not cell confluence per se appears to be a prerequisite for adipocyte differentiation (Gregoire et al. 1998). Our results showed that

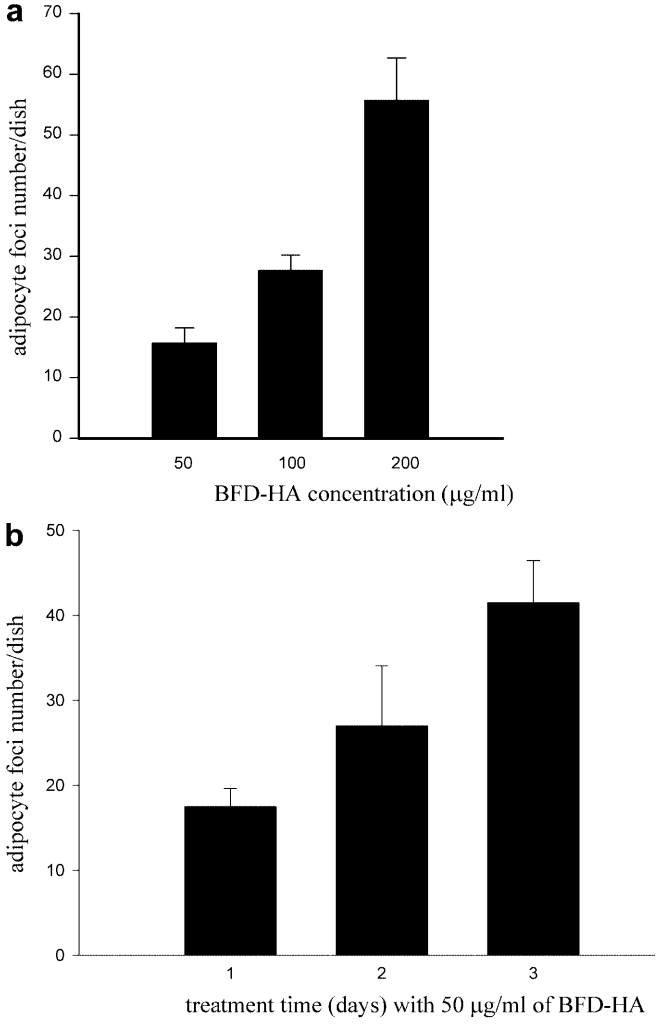


**Fig. 3** The adipocyte foci number of C3H10T1/2 cells increased dose-dependently after treatment with BFD-HA. C3H10T1/2 cells were treated with BFD-HA at 2 days confluence and remained in culture for 2 weeks. Then the adipocyte foci numbers were counted. (**a**) C3H10T1/2 cells were treated with 50, 100 and 200 μg/ml of BFD-HA for 24 h. (**b**) C3H10T1/2 cells were treated with 200 μg/ml of BFD-HA for 1, 2 or 3 days. Data are shown as means ± SD of three independent experiments

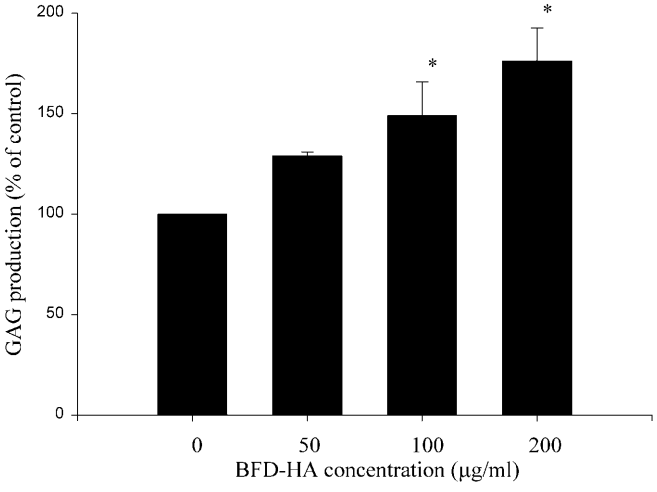


**Fig. 4** The synthesis of GAG was increased by BFD-HA in C3H10T1/2 cells. C3H10T1/2 cells were treated with various concentrations of BFD-HA at 2 days confluence. The control group is set as 100%. Data are shown as means ± SD of three separate experiments. *$P < 0.05$ compared to each control

C3H10T1/2 cells rapidly underwent growth arrest after 24 h treatment with BFD-HA. The distribution of the cell cycle revealed that about 80% of cells were arrested at the $G_1$ phase after incubation with 200 μg/ml BFD-HA for more than 9 h. In addition to C3H10T1/2 cells, we and another laboratory previously reported that humic acid induces $G_1$-phase arrest in diverse cells, including human colon adenocarcinoma HT 115 cells, human embryonic lung fibroblast LEP cells, human myelomonocytic leukaemia U937 cells (Hofmanová et al. 1999), and mice Sertoli TM4 cells (Chen et al. 2001). TM4 cells were retarded at the $G_1$ phase by humic acid via the decrease in cyclin D1 expression and increased $p27^{kip1}$ expression. Recently, we also found that the growth arrest of 3T3-L1 pre-adipocyte cells induced by humic acid was mediated by a decline in cyclin D1 expression and increment in p21 expression (unpublished data).

During the terminal phase of differentiation, adipocytes exhibit marked increases in de novo lipogenesis and numbers of glucose transporters and insulin receptors increase as does insulin sensitivity. Before activation of the adipogenic-specific genes, the mRNA for PPAR $\gamma$ appears (Smas et al. 1995). It is now accepted that PPARs not only play a crucial role in the intracellular lipid metabolism but are also an important regulator of extracellular lipid metabolism (Schoonjans et al. 1996). Besides in adipose tissue with adipogenic potentials, PPARs are also expressed in atherosclerotic lesions, including the subendothelial region and in the lipid core where they co-localise with specific markers of macrophages, smooth muscle cells and foam cells. Via negative regulation of nuclear factor-kappa B and activator protein-1 signalling pathways, PPARα inhibits expression of inflammatory genes, such as interleukin-6, cyclooxygenase-2 and endothelin-1. PPARγ activator in macrophages and foam cells inhibits the expression of activated genes such as inducible nitric oxide synthase, matrix metalloproteinase-9 and scavenger receptor A. PPARγ may also affect the recruitment of monocytes in atherosclerotic lesions as it is involved in the expression of vascular cell adhesion molecule (VCAM)-1 and intracellular adhesion molecule-1 in vascular endothelial cells (Chinetti et al. 2001). Being a peroxisome proliferator, humic acid could induce the expression of PPARα and γ in rodent cells (Lee et al. 1999). It is possible that the large lipid accumulation in the arterial wall of BFD patients is mediated by the regulation of PPAR α and γ. However, more investigations will be needed to solve the problem.

The molecular events leading to the determination of C3H10T1/2 cells to the adipocyte lineage remain to be characterised. In the adipocyte differentiation model of C3H10T1/2 cells, the cells have to undergo the two steps includes determination and differentiation and then complete the process of adipocyte conversion. Moreover, the surrounding cells of the determinated cells usually ameliorate adipocyte differentiation simultaneously and then form the adipocyte foci. To date, the formation of the adipocyte foci is considered to result from the cell-cell interaction or division from the same cell (Gregoire et al. 1998). That is why that the adipocyte foci were observed but not all C3H10T1/2 cells express the adipocyte phenotype after treatment with BFD-HA.

Several lines of evidence suggest that a number of the biological manifestations of atherosclerosis are due to changes in arterial extracellular matrix (Edwards et al. 1995). Rapidly proliferating arterial smooth muscle cells from atherosclerosis-susceptible pigeons are associated with decreased heparan sulphate proteoglycan. Chondroitin-4-sulphate proteoglycan is a major component of atherosclerotic lesions and a correlation between apo B and artery chondroitin-4-sulphate content has been demonstrated in the lesions. Our present data show that the GAG production was increased after 24 h of treatment with 200 μg/ml BFD-HA in C3H10T1/2 cells. This suggests that such changes in the extracellular matrix might be in the central scene of the formation of atherosclerotic-like plaque in BFD patients.

In conclusion, our data demonstrate that BFD-HA induces C3H10T1/2 cell growth arrest at the $G_1$ phase, and then differentiation to adipocyte. The GAG production of C3H10T1/2 cells was also increased by BFD-HA simultaneously. Our findings suggest that the adipocyte conversion of fibroblasts cells may play a significant role in the formation of atherosclerotic-like plaque in BFD patients.

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