Integrin β4 mAb inhibited apoptosis induced by deprivation of growth factors in vascular endothelial cells[1]

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KEY WORDS integrin 4; monoclonal antibodies; apoptosis; umbilical veins; vascular endothelium; cyclic AMP; protein p53; *ras* genes

ABSTRACT

AIM: To understand the mechanism by which anti-β4 integrin monoclonal antibody (mAb) inhibits apoptosis of vascular endothelial cells (VEC). **METHODS:** Viability was determined by counting the cells that attached to dishes after treatments. DNA fragmentation was analyzed by agarose gel electrophoresis and fluorescence microscopy. The intracellular content of cAMP was measured by radioimmunoassay (RIA). The levels of p53 and Ras expressions were analyzed by fluorescence microscopy combined with immunofluorescence under laser scanning confocal microscopy. **RESULTS:** After the cells were deprived of fibroblast growth factor (FGF) and serum were exposed to the mAb 5 mg/L for 24 h, the detachment and DNA fragmentation of these cells were suppressed. When cells were deprived of FGF and serum, the intracellular cAMP level and Ras protein content decreased ($P<0.05$), while the level of p53 protein expression increased ($P<0.05$). But in the presence of anti-β4 integrin mAb, VEC apoptosis was inhibited, and at the same time, the changes mentioned above were obviously blocked ($P<0.05$). **CONCLUSION:** Anti-4 integrin mAb inhibited apoptosis by affecting the level of cAMP, and blocking down-regulation of Ras protein and up-regulation of p53 protein in VEC.

INTRODUCTION

The integrin α6β4 is a receptor for the laminin family of extracellular matrix proteins. In addition to serving a significant structural function in the assembly of hemidesmosomes in epithelial cells[1,2], α6β4 promotes carcinoma cell migration and invasion[3,4]. The diverse activities of this integrin are exemplified by its ability to trigger both the survival of keratinocytes and the apoptosis of a number of carcinoma cell lines[4, 5]. These apparently contradictory functions may reflect the activation of different signaling pathways by this integrin in different cell types as well as the influence of other signaling pathways on α6β4 function, suggesting that the functions of α6β4 are cell type-specific.

In rectal carcinoma cells, overexpression of the cytoplasmic domain of β4 integrin can induce apoptosis by the activation of the p21 (WAF/Cip1) pathway[6], but in A431 cancer cells, the β4 integrin subunit rescues the cells from apoptosis through a PI3K/Akt kinase signaling pathway[7]. In normal vascular endothelium cells (VEC) that expressed integrin β4, the apoptosis induced by deprivation of FGF and serum was obviously inhibited by anti-β4 integrin mAb[8], but the mechanism by which anti-β4 integrin mAb inhibitsed apoptosis of VEC was not clear.

Integrin β4 subunit might be more critical in

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apoptosis signal transduction than α6 subunit because α6 antibody had no effect on cell survival[6,7]. In this study, we used the mAb of integrin β4 subunit and studied the possible mechanism by which this integrin regulated apoptosis of VEC.

On the bases of our previous findings[8], cAMP level, p53 and Ras protein expressions at the early stage of apoptosis were examined in the absence or presence of the mAb in VECs deprived of FGF and serum to understand the mechanism of mAb against integrin β4 inhibiting apoptosis in VEC.

MATERIALS AND METHODS

Reagents M199 medium was purchased from Gibco BRL Co, Grand Island, NY. Fetal bovine serum (FBS) was purchased from Hyclone Lab Inc USA. Fibroblast growth factor (FGF) was extracted from bovine brains by the method of Lobb and Fett[9]. Anti-β4 integrin mAb (clone 3E1) was bought from Chemicon International Inc, Temecula, USA. An irrelevant mAb (mouse IgG) was used as a control, and mouse IgG at the same concentration used in this paper did not show any effect on the cells. The ^{125}I-labeled cyclic AMP radioimmunoassay kit (cat#1117) was purchased from Immunotech, a Beckman Coulter Company, France. Rabbit anti-human P53, a rabbit polyclonal antibody raised against a peptide mapping at the middle region of P53 of human origin, anti-H-Ras, a rabbit polyclonal antibody raised against a peptide corresponding to 168-181 of H-Ras of human origin identical to the related rat sequence, and SABC-FITC immunohistological staining kits were purchased from Wuhan Boster Biological Technology Co Ltd, China.

Cell culture Human umbilical VEC were obtained by the method of Jaffe *et al*[10]. The cells were cultured in gelatin-coated plastic dishes in M199 medium, supplemented 10 % fetal bovine serum (FBS), 70 μg/L FGF (as well as 40 mg/L heparin) at 37 ºC in a humidified atmosphere of 5 % CO_2 and 95 % air. Cells with a population doubling level of 15-30 were used.

Viability assay The experiments were performed as described previously[11]. Briefly, when cultured cells reached confluence, the cells were washed once with M199 medium and replaced with the medium. The cells were incubated with or without anti-β4 integrin mAb 5 mg/L. Trypsinized cells were counted with a Coulter counter after 24 h and 48 h. Detached cells were washed away before the treatment with trypsin. The cells that remained attached to dishes after washing away of blebs were not stained by erythrosine B (5 g/L, Sigma) and were therefore, regarded as living cells.

Analysis of cAMP level Intracellular cAMP assay was performed as described previously[12]. Briefly, 1×10^6 cells had been treated with or without anti-4 integrin mAb 5 mg/L for 6 h. The cAMP was measured by radioimmunoassay according to the manufacturer's recommendation. The cAMP level was expressed as nanomol per 1×10^6 cells.

Analysis of p53 and Ras protein expressions Fluorescence microscopy combined with immunocytochemistry was performed in accordance with previous studies[13-15]. Briefly, the cells treated with or without anti-β4 integrin mAb 5 mg/L for 3 h or 6 h were washed with PBS 0.1 mol/L, fixed with 4 % paraformaldehyde for 10 min. After the treatments of primary antibody and secondary antibody, the samples were photographed and the expression levels of P53 and Ras expression were quantified under laser scanning confocal microscopy (Carl Zeiss LSM510). The levels of p53 protein and Ras protein were expressed as relative fluorescence intensity.

Statistical analysis Data were expressed as mean±SD and analyzed by *t*-test. $P<0.05$ was considered to be statistically significant.

RESULTS

Inhibitory effect of anti-β4 integrin mAb on apoptosis in VEC When VECs were deprived of FGF and serum from M199 medium, some cells gradually detached from the dish and apoptosis occurred. After VEC was exposed to anti-β4 integrin mAb 5 mg/L in the absence of FGF and serum for 24 h, VEC apoptosis was inhibited (Fig 1, 2). The viability was higher than that of untreated cultures ($P<0.01$, Tab 1).

Effect of mAb against integrin β4 on level of cAMP In the cells cultured in the medium with FGF and serum, cAMP level was 55±6 nmol/L per 1×10^6 cells. When the cells were deprived of FGF and serum, apoptosis occurred, and at the same time the content of cAMP of these cells decreased to 28±6 nmol/L per 1×10^6 cells. After VEC apoptosis was inhibited by anti-β4 integrin mAb, the intracellular cAMP level rose to 57±7 nmol/L per 1×10^6 cells ($P<0.05$).

Effect of mAb against integrin β4 on expression of p53 protein After a deprivation of FGF and serum for 3 h and 6 h, the levels of p53 protein were higher than those in normal growth cells cultured in the medium with FGF and serum ($P<0.05$). When

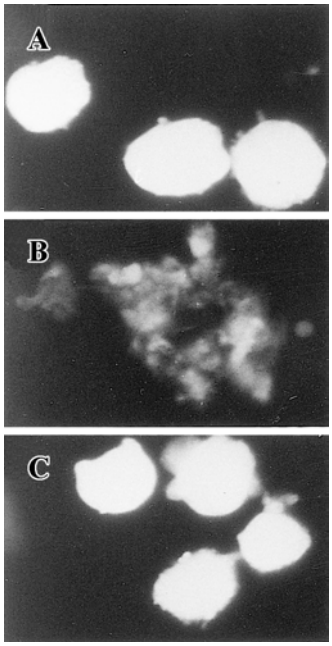

Fig 1. Nuclear fragmentation of VEC stained with Hoechst 33258 at 24 h. (A) The nuclei of cells cultured in the medium with FGF and serum; (B) The nuclei of cells deprived of FGF and serum; (C) The nuclei of cells treated with the mAb 5 mg/L in the absence of FGF and serum. ×1000.

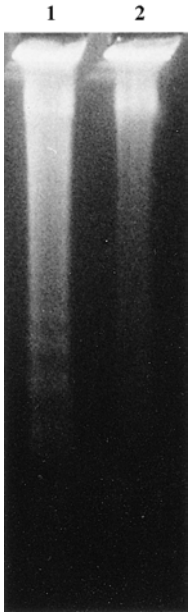

Fig 2. Effect of anti-β4 integrin mAb on DNA fragmentation in apoptosis of VEC at 24 h. 1) DNA from cells deprived of FGF and serum; 2) DNA from cells treated with the mAb 5 mg/L.

apoptosis of VEC was inhibited by the mAb, the content of p53 protein considerably decreased ($P<0.05$) after the 3-h and 6-h treatments (Fig 3).

Tab 1. Time course of effect of mAb against integrin β4 5 mg/L on VEC apoptosis. The cells remaining on the dishes were counted at 24 and 48 h after the start of treatment. n=5. Mean±SD. $^bP<0.01$ *vs* control.

Time/h	Viability %	
	Control	mAb-treated
24	60.2±1.5	97.1±2.0[b]
48	23.7±2.2	68.3±1.8[b]

Effect of mAb against integrin β4 on expression of Ras protein After the deprivation of FGF and serum for 3 h and 6 h, the levels of Ras protein were obviously lower than those in normal growth cells cultured in the medium with FGF and serum ($P<0.05$). When apoptosis of VEC was inhibited by anti-β4 integrin mAb, the content of Ras protein considerably increased ($P<0.05$) after the 3-h and 6-h treatments (Fig 4).

DISCUSSION

Human VEC are absolutely dependent upon the presence of FGF and serum for proliferation and serial propagation *in vitro*[16], and in the presence of FGF, the expression of integrin β4 was obviously suppressed. When the cells were deprived of FGF and serum from the culture medium, the level of integrin β4 mRNA increased[17]. So VEC is a very useful model for investigating the role of integrin β4 in apoptosis signaling of normal cells. The mAb of integrin β4 used in this study is specific to human β4 integrin[18], while normal mouse IgG used as an antibody control had no effect on VEC[8].

The classical second messenger molecule cyclic AMP dramatically modulates the apoptotic program in a variety of cells, accelerating apoptosis in some and delaying the rate of apoptosis in others[19]. To determine whether cAMP is implicated in the signal transduction pathway mediated by integrin β4, we treated the cells with or without the mAb in the absence of FGF and serum. The present results confirmed that integrin β4 mediated the signal transduction by affecting cAMP level in VEC.

In our previous study, it was shown that the mRNA level of p53 gene increased when VEC apoptosis was induced by deprivation FGF and serum. This indicated that p53 gene might play an important role in apoptosis of VEC[20]. In this study, to understand

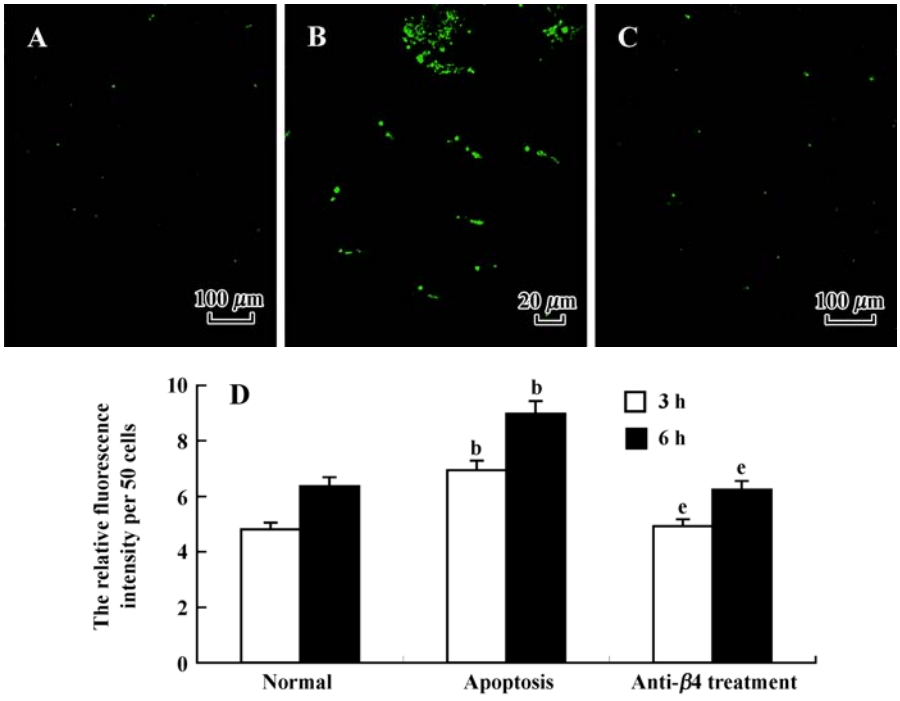

Fig 3. Effect of mAb against integrin β4 on the expression of p53 protein. The location and the relative intensity of p53 in the cells cultured in the medium with FGF and serum (A), deprived of FGF and serum (B), and treated with the mAb 5 mg/L in the absence of FGF and serum respectively (C). (D) The quantity of expressed p53 from the cells. *n*=3. Mean±SD. $^{b}P<0.05$ *vs* normal control. $^{e}P<0.05$ *vs* apoptosis group.

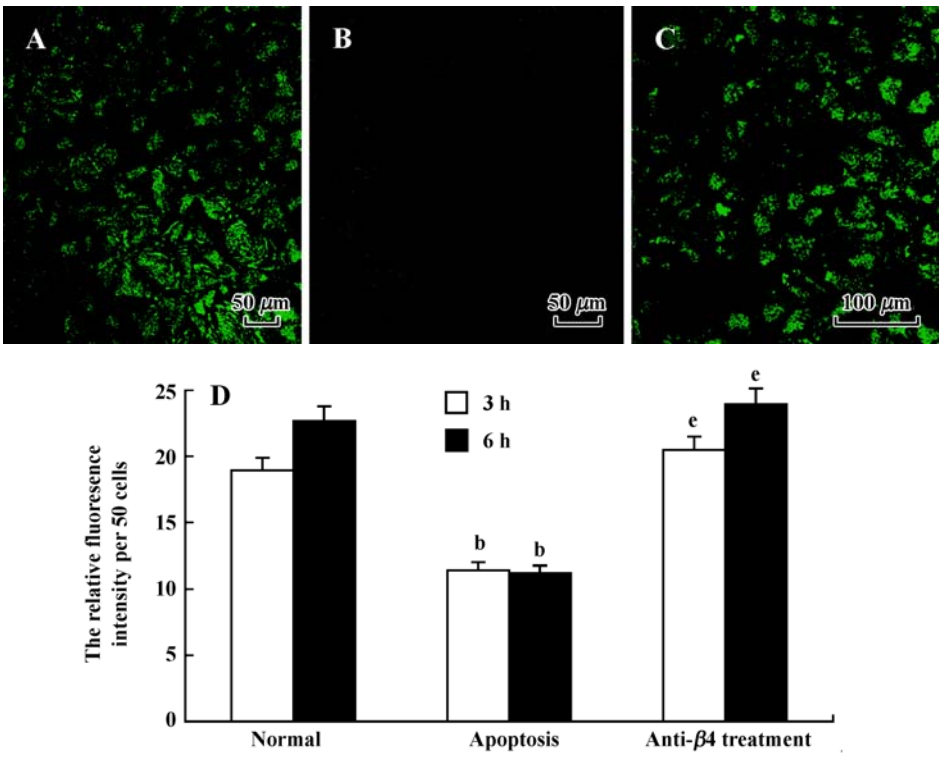

Fig 4. Effect of mAb against integrin β4 on the expression of Ras protein. The location and the relative intensity of Ras in the cells cultured in the medium with FGF and serum (A), deprived of FGF and serum (B), and treated with the mAb 5 mg/L in the absence of FGF and serum respectively (C). (D) The quantity of expressed Ras from the cells. *n*=3. Mean±SD. $^{b}P<0.05$ *vs* normal control. $^{e}P<0.05$ *vs* apoptosis group.

whether integrin β4 mediates apoptotic signal by regulating p53 expression, we examined the expression of p53 protein when VEC apoptosis was inhibited by the antibody of this integrin. The results showed that integrin β4 mediated apoptotic signal transduction by upregulating p53 protein expression.

Ras is another important protein that is related to cell growth and apoptosis. The growth-suppressive function of wild-type p53 can be bypassed by activation of the ras gene[21]. In the previous research, we found that at 3 h after deprivation of FGF and serum, the level of *c-H-ras* mRNA was half of that in control cells cultured in the medium with FGF and serum, indicating that the c-H-ras might be involved in the apoptotic process of VEC[22]. To address the possibility that integrin participates in regulation of the expression of Ras protein in VEC apoptosis, in this study, the cells that were deprived of FGF and serum were treated with anti-β4 integrin mAb. The results showed that integrin β4 mediated apoptotic signal transduction by down-regulating Ras protein expression. The data demonstrated that when the function of integrin β4 was blocked by its specific antibody, the Ras expression was promoted.

In summary, we presented evidence that, at the early stage of VEC apoptosis, anti-β4 integrin mAb inhibited apoptosis by affecting the level of cAMP, down-regulating p53 protein expression and up-regulating the expression of Ras protein at the same time. These findings should facilitate our understanding of the complicated mechanisms by which integrin β4 regulates apoptosis signaling. The findings also provided evidence for the clinical application of anti-β4 integrin mAb.

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