S-phase delay in human hepatocellular carcinoma cells induced by overexpression of integrin β1

Yu-Long Liang, Ting-Wen Lei, Heng Wu, Jian-Min Su, Li-Ying Wang, Qun-Ying Le, Xi-Liang Zha

AIM: To clarify the mechanisms of integrin overexpression in negatively regulating the cell cycle control of hepatocellular carcinoma cells SMMC-7721.

METHODS: The cell cycle pattern was determined by flow cytometry. The mRNA and protein expression levels were assayed by RT-PCR and Western blot, respectively. Stable transfection was performed by Lipofectamine 2000 reagent, and cells were screened by G418.

RESULTS: Overexpression of α5β1 or β1 integrin induced S-phase delay in SMMC-7721 cells, and this delay was possibly due to the accumulation of cyclin-dependent kinase inhibitors (CKIs) p21\(^{kip1}\) and p27\(^{kip1}\). The decrease of protein kinase B (PKB) phosphorylation was present in this signaling pathway, but focal adhesion kinase (FAK) was not involved. When phosphorylation of PKB was solely blocked by wortmannin, p27\(^{kip1}\) protein level was increased. Moreover, S-phase delay was reversed when attachment of the parental SMMC-7721 cells was inhibited by the preparation of polyHEMA, and this cell cycle pattern was similar to that of β1-7721 or α5β1-7721 cells.

CONCLUSION: S-phase delay induced by overexpression of integrin β1 subunit is attributed to the decrease of PKB phosphorylation and subsequent increases of p21\(^{kip1}\) and p27\(^{kip1}\) proteins, and may be involved in the unoccupied α5β1 because of lack of its ligands.

Liang YL, Lei TW, Wu H, Su JM, Wang LY, Lei QY, Zha XL. S-phase delay in human hepatocellular carcinoma cells induced by overexpression of integrin β1. World J Gastroenterol 2003; 9(8): 1689-1696

http://www.wjgnet.com/1007-9327/9/1689.asp

INTRODUCTION

Extracellular matrix (ECM) is consisted of many components, such as collagen, glycoproteins, elastin, and proteoglycans that in addition to providing a scaffold for tissue, regulate many fundamental cellular processes such as proliferation, survival, migration and differentiation\[1,2\]. Many cell types require anchorage to ECM to proliferate\[3\]. If lacking attachment to ECM, they will undergo anoikis\[4,5\]. Integrins activate growth-promoting signaling pathways that are responsible for the anchorage, and two such pathways appear to be involved. One is that integrins facilitate growth factor-mediated activation of extracellular signal-regulated protein kinase (ERK); the other is that integrins activate the c-Jun NH\(_2\)-terminal kinase (JNK)\[6,7\]. In addition, serine/threonine protein kinase, PKB, has emerged as a crucial regulator in the integrin pathway, which can be controlled through phosphatidylinositol-3' kinase (PI3K)\[8]. Integrins, often together with growth factor receptors, up-regulate cyclins D and E and down-regulate CKIs p21\(^{cip1}\), p27\(^{kip1}\), and p57\(^{kip2}\)\[9,10\]. This action allows cells to pass through the G1/S transition and complete the cell cycle.

Many studies, however, have demonstrated that integrins give rise to growth inhibition rather than growth stimulation\[11,12\]. Integrin α5β1 has been often observed to be lost in cancerous areas other than in its normal counterpart tissues\[13\]. It is apparent from these studies that integrin signaling may play a major role in negative control of cell growth, which may be lost in some cancer cells, and the mechanisms of this effect are not completely known yet.

In this study we have further investigated the inhibition role of integrin α5β1 in human hepatocellular carcinoma cell line, SMMC-7721. We analyzed the effect of these cells with or without adhesion to ECM. These studies identified overexpression of β1 subunit or α5β1 inhibited cell cycle progression at S-phase, and this inhibition maybe resulted from the up-regulation of cdk2 inhibitors p21\(_{up}\) and p27\(_{up}\) and involved in the unoccupied β1 because of relative lack of its ligands.

MATERIALS AND METHODS

Materials and antibodies

Poly-HEMA, wortmannin, FN and LN were all obtained from Sigma, and genetin (G418) was purchased from Calbiochem (San Diego, CA). Monoclonal antibodies used were directly against cyclin D1 (Santa Cruz), cdk2 (Santa Cruz), integrin β1 (BD Transduction Laboratories, Lexington, KY), phosphorylated FAK (anti-phosphotyrosine clone PT-66, Sigma), and β-actin (Santa Cruz). Goat anti-human integrin α5 polyclonal antibody was also purchased from Santa Cruz. Other antibodies used were those against FAK (Santa Cruz), p21\(_{up}\) (Santa Cruz), p27\(_{up}\) Oncogene Research Products, Cambridge, MA), Ser473-phosphorylated form of PKB and PKB (Cell Signaling). Horseradish peroxidase conjugated anti-mouse, rabbit or goat IgG were purchased from Calbiochem (San Diego, CA).

Cell culture

Human hepatocellular carcinoma cell line SMMC-7721 was obtained from the Liver Cancer Institute, Zhongshan Hospital (Shanghai, China). SMMC-7721 cells were grown in RPMI 1640 medium (Gibco BRL) supplemented with 100 mL·L\(^{-1}\) calf bovine serum (CBS), 100×10\(^{3}\) U·L\(^{-1}\) penicillin and 100×10\(^{3}\) U·L\(^{-1}\) streptomycin sulfate. Integrin-overexpressing transfectant
cell lines were maintained in the same medium as above plus 500 µg/ml genicin (Gibco BRL).

**Plasmid construction and stable transfections**

pECE vector containing human full-length cDNA of integrin β1 was presented generously by Dr. Mara Brancaccio (Department of Genetics, University of Torino, Torino, Italy). Complementary DNA of β1 integrin cleaved from the pECE plasmid by EcoRI I was subcloned into pcDNA3 vector to generate pcDNA3-β1. pcDNA3-α5 expression vector was presented kindly by Dr. Sue E. Craig (School of Biological Sciences, University of Manchester, Manchester, UK). Stable transfections were performed using LIPOFECTAMINE™ 2000 (LF2000) reagent (Life Technologies, Grand Island, NY) according to the manufacturer’s instructions. Briefly, logarithmically growing cells were transfected with 1 µg of plasmids and 2 µl of LF2000 reagent. At 48 h after transfection, the selective medium containing 1 mg/ml genicin (G418) antibiotic (Calbiochem) was replaced, and then the cell clones were selected and identified.

**Plating experiments**

Mock and integrin transfected cells were seeded on the tissue culture plates coated with either FN (15 µg/ml) or LN (15 µg/ml), grown for 60 minutes, and followed by phase-contrast microscopy or protein extraction.

**Flow cytometry**

Cells were starved by exposure to SFM for 48 h, the harvested cells were then grown in normal growth medium containing 10% FBS for 12-16 h, the time span covered the duration of normal S phase. At the end of incubation, the cells were digested with 2 mM EDTA in PBS and rinsed twice with ice-cold PBS solution, then fixed by adding them dropwise into 75% ice-cold ethanol for 37°C for 30 min in 0.5 ml PBS solution containing 20 µg/ml RNase A, 0.2% Triton X-100, 0.2 mM EDTA and 20 µg/ml of propidium iodide. DNA content was determined by FACS analysis (Becton Dickinson). The percentage of cells in G0/G1, S, and G2/M phases was determined using the Modfit program.

**RNA isolation and RT-PCR**

RT-PCR was performed to quantify the level of mRNA, which was isolated using Trizol system (Watson Biotechnologies, Shanghai, China) according to the manufacturer’s guidelines. Complementary DNA synthesis was performed essentially as described previously,[15] except that 2 µg of total RNA was used for cDNA synthesis, and the primer used was oligo (dT). For amplification, 2-µl cDNA product was used in a final volume of 50 µl with 5 units of Taq polymerase (SABC, Luoyang, China). The primer pairs for p27[16] and p21[17] were described previously. Primers for β-actin[16] were used as the internal control. The expected product sizes were p27, 471 bp; p21, 159 bp and β-actin, 412 bp.

**Cell lysis and immunoblotting**

Cells cultured under the same conditions as cell cycle analysis were collected and then washed twice with ice-cold PBS and lysed in 1xSDS lysis buffer (50 mM Tris (pH 6.8), 2% SDS, 10% glycerol, 100 µg/ml PMSF, 10 µg/ml leupeptin and 5 mM Na3VO4) for 10 min on ice. Cell lysates were boiled and clarified by centrifugation at 12000 g at 4°C for 10 min. Protein concentration was determined with Hartree assay. Immunoblotting analyses using the enhanced chemiluminescence (ECL) detection system (Perfect, Shanghai, China) were carried out as described previously.[18]

**RESULTS**

**Overexpression of integrin α5β1 in SMMC-7721 cells**

We transfected the full-length cDNA of genes ITGA5 (α5) or ITGB1 (β1) alone, or α5 and β1 together into a human hepatocellular carcinoma cell line, SMMC-7721, respectively. The pcDNA3 empty vector was the control plasmids, and cells transfected with pcDNA3 were regarded as the mock cells. The overexpressed transfecant cell lines were mainly screened for increased expression of α5 or β1 in protein levels by Western analysis, and designated as α5-7721, β1-7721 and α5β1-7721, respectively. As shown in Figure 1A, integrin α5 expression was increased to 2-fold in α5-7721 or α5β1-7721 cells compared with the mocked cells. Meanwhile, β1-7721 and α5β1-7721 transfectants had more than 2.5-fold amount in integrin β1 protein level (Figure 1B). The β1 subunits appeared as two bands in Western blot because of variable post-translational modification (mainly N-glycosylation). The hypoglycosylated lower band (Figure 1B) was tentatively identified as biosynthetic precursor of β1 subunit. The band with lower migration rate (upper band in Figure 1B) of integrin β1 subunit was in hyperglycosylated forms, and mainly located in plasma membrane[16-21].

![Figure 1: Integrin α5 and β1 protein levels in α5-, β1- and α5β1 transfected SMMC-7721 cells.](image-url)
Figure 2 S-phase delay was induced in β1- and α5β1-transfectant cells. For cell cycle analysis, transfected and mocked cells were synchronized by exposure to SFM for 48 h, then grown in RPMI1640 medium containing 10% CBS and penicillin/streptomycin solution. Twelve or 16 h later, the cells were collected and analysed for flow cytometry as described under “Materials and Methods”. A, mocked cells; B, α5-7721 cells; C, β1-7721 cells; D, α5β1-7721 cells. Each bar in graph represented the mean ± SD obtained from three independent experiments. The S-phase delay was significantly different in β1-7721 and α5β1-7721 cells (n=3, *P < 0.01 vs mocked cells).

Figure 3 Message RNA and/or protein levels of cell cycle regulatory genes p21cip1 and p27kip1 in β1-7721 and α5β1-7721 transfectants, but not that of cyclin D1 and cdk2. (A) Immunoblot assay showed the protein level of cyclin D1 and cdk2 were not apparently affected, but p21cip1 and p27kip1 protein levels were increased in β1-7721 and α5β1-7721 cells. The protein level of β-actin was detected to assess the loading amount in each well in SDS-PAGE gel. (B) Message RNA levels of p21cip1 and p27kip1 were assessed by RT-PCR, and normalized by that of β-actin. It was apparent that mRNA level of p21cip1 was increased in β1- and α5β1-transfected cells. However, the p27kip1 mRNA amount was the same as control. Each result represented three separate experiments. Lane 1, mocked cell; lane 2, α5-7721; lane 3, β1-7721; lane 4, α5β1-7721.
Induction of S-phase delay by transfection of integrin β1 subunit

In the previous study, we showed that overexpression of integrin α5β1 or β1 subunit had negative effects on cell growth[13]. To elucidate the mechanisms of cell cycle perturbation induced by overexpressing integrins, flow cytometry analyses were applied. Cell cycle parameters were compared between transfected cells and mocked cells. Similar patterns of the cell cycle were found in α5-7721 and mocked cells (Figure 2). But in β1-7721 and α5β1-7721 transfectants, we observed a significant increase in fraction of cells in S phase of the cell cycle, as shown in Figure 2. This was accompanied by a decrease in proportion of cells in G2/M phases of the cell cycle. These changes were specific for the transfection events containing β1-plasmids, that is, at this point, the pattern of α5β1-7721 was similar to that of β1-7721 cells. These results were obtained from the cells synchronized partly in G0/G1 phase by exposure to serum-free medium. Therefore, these data showed that S-phase delay was probably due to enhanced production of exogenous β1 integrin.

p21<sup>kip1</sup> and p27<sup>kip1</sup> were up-regulated in β1 or α5β1 transfectant cell lines

To clarify the mechanism by which S-phase delay was induced in β1-7721 and α5β1-7721 cells, we investigated whether cyclins and cdks were involved in this situation. Expression level of cyclin D1 was examined as a sensor molecule to the extracellular cues[22]. It was shown that cyclin D1 expression was not changed, neither was the cdk2 protein (Figure 3A). As evidenced recently, enhanced p21<sup>kip1</sup> and/or p27<sup>kip1</sup> expression was considered to be associated with G1 cell cycle arrest[23, 24], and under some circumstances, with S-phase delay in some cell types[25, 26]. So, we further examined whether β1-chain overexpression could induce p21<sup>kip1</sup> and p27<sup>kip1</sup> in β1-7721 and α5β1-7721 cells. As shown in Figure 3A, a significant increase (2-fold amount) of p21<sup>kip1</sup> protein levels was noted in β1-7721 and α5β1-7721 cells compared with the mocked cells or α5-7721 cells. Meanwhile, p27<sup>kip1</sup> protein level was also increased to 2.5-fold. We also found that the mRNA level of p21<sup>kip1</sup> increased, although that of p27<sup>kip1</sup> maintained the same as control (Figure 3B). Interestingly, we found that overexpression of β1 gene in SMMC-7721 cells induced S-phase delay in this study. The above findings showed that this S-phase delay might be attributed to the increased expression of p21<sup>kip1</sup> and p27<sup>kip1</sup>.

Phosphorylated form of PKB was inhibited in β1 and α5β1 transfectants and this might result in accumulation of p21<sup>kip1</sup> and p27<sup>kip1</sup>

To determine how overexpressing α5β1 integrins transferred signals from membrane to cytosol or nucleus to modulate the expression of CKIs p21<sup>kip1</sup> and p27<sup>kip1</sup>, we examined the two important signaling molecules mediated by integrins, FAK and PKB. The results showed that neither FAK nor its tyrosine phosphorylated form was affected (Figure 4A). However, levels of Ser473-phosphorylated form of PKB were decreased in β1-7721 and α5β1-7721 cells in comparison with the control cells, but total amount of PKB protein was not apparently affected (Figure 4B).

In recent years, evidences have shown that activated PKB protein may phosphorylate p21<sup>kip1</sup>, and induce its degradation through the ubiquitin-26S proteasome pathway[6, 27]. But evidences indicating p27<sup>kip1</sup> phosphorylation by PKB are few. In this study, decrease of phosphorylated PKB (Figure 4B) and accumulation of p27<sup>kip1</sup> (Figure 3A) occurred concomitantly in β1-7721 or α5β1-7721 cells. To obtain support, we further performed an experiment to block the phosphorylated form of PKB. It is well known that Ser-473 phosphorylation of PKB, on behalf of its active forms, appears to be catalyzed by phosphoinositide-dependent kinase 1 (PDK1) and integrin-linked kinase (ILK)[28, 29], and that PI3K is located upstream of both ILK and PKB[30]. So the PI3K inhibitor wortmannin was explored in this study. Following serum starvation, cells were cultured in the medium with 100 nM wortmannin for 24 h or 48 h. We investigated the level of phosphorylation of PKB and p27<sup>kip1</sup> by immunostaining. The data showed that phosphorylation of PKB was blocked, and expression of p27<sup>kip1</sup> was increased at the same time compared with control cells (Figure 5). Therefore, the decrease of phosphorylated form of PKB was at least in part, responsible for p27<sup>kip1</sup> protein accumulation.

Figure 4 Activation of PKB, but not FAK, was downregulated in β1- and α5β1 transfectants. (A) FAK activation was assessed by phosphotyrosine-specific antibody, followed by stripping and reprobing with anti-FAK antibody. (B) PKB and its Ser473-phosphorylated forms were determined by 10% SDS-PAGE with the equal loading amount. The loading amount control is shown in Figures 1C and 3A. Lane 1, mock cells; lane 2, α5-7721; lane 3, β1-7721; lane 4, α5β1-7721. Results were representative of 4 repeated experiments.

Figure 5 Ser473-phosphorylated form of PKB was decreased, but p27<sup>kip1</sup> protein level was increased concomitantly in SMMC-7721 parental cells treated with the PI3K inhibitor wortmannin. The parental SMMC-7721 cells were starved with serum-free medium for 48 h, then grown in normal medium/10% CBS containing DMSO (as control, 24 h) or 100 nM wortmannin for
the indicated times. The amount of DMSO did not exceed 0.1 %, which was determined not to damage the cells. Equal amount of wortmannin was added again after grown for 24 h. The level of Ser473-phosphorylated form of PKB (arrow) was declined, but p27kip1 protein level was elevated with the treatment of wortmannin in SMMC-7721 cells. Results were representative of at least 3 repeated experiments. Abbreviation: DMSO, dimethylsulfoxide; WT, wortmannin.

**Inhibition of cell cycle was possibly due to the relative lack of ECM**

Integrins, in general, promote the focal adhesion protein activities such as FAK and cell cycle progression[2]. So we ponder why overexpression of integrin β1 subunit can repress the level of phosphorylated PKB and the cell cycle. Here, we performed plating experiments to elucidate its mechanism. First, we observed the morphological change of cells that were plated on LN- or FN- coated dishes and grown for 60 minutes (Figure 6). It was found that β1-7721 and α5β1-7721 cells were prone to attachment and spreading, perhaps to the cell cycle progression. Next, we determined the changes of phosphorylated FAK and phosphorylation of PKB in cells attaching on LN and FN. As mentioned above (Figure 4A), total amount of FAK and its phosphorylated forms were not affected in cells cultured in the flasks without coating of LN or FN. But the level of tyrosine phosphorylated FAK was increased in cells, which were plated on LN- or FN- coated dishes and grown for the indicated times (Figure 7A, and data not shown for FN-coated dishes). Moreover, the level of Ser473 phosphorylated form of PKB was similar to that of phosphorylated FAK under the same condition (Figure 7B, and data not shown for FN-coated dishes). Finally, protein level of p27kip1 was declined in β1-7721 and α5β1-7721 cells (Figure 7C). These findings demonstrated that an important role of LN or FN in the molecular changes of β1-7721 or α5β1-7721 cells, especially, in PKB phosphorylation and p27kip1 protein levels. On the contrary, when attachment of the parental cells to ECM was blocked by plating them onto poly-HEME-coated petri dishes, the percentage of cells in S-phase was increased from 13.08 % to 37.33 % (Figure 8). That is, when the parental SMMC-7721 cells were prevented from interaction with ECM through the preparation of poly-HEME, the same effects of S-phase accumulation took place as that in β1-7721 or α5β1-7721 cells. These results suggested that S-phase delay induced by overexpressing β1 in SMMC-7721 cells might be the result of the relative lack of ECM.

**Figure 6** β1-7721 and α5β1-7721 cells subjected to spreading on fibronectin- or laminin- coated culture dishes. The mocked and transfected cells were plated on FN- or LN- coated tissue culture plates in normal medium for 60 min, then cells on the plates were photographed by phase-contrast microscopy with a digital camera. Abbreviations: FN, fibronectin; LN, laminin.
Figure 7 Effects of laminin on transfected cells. Protein levels of phosphorylated FAK (A) and Ser473-phosphorylated form of PKB (B) were increased in the transfected cells, especially in β1-7721 and α5β1-7721 cells grown in the LN-coated culture dishes for 60 minutes. (C) Under the same condition, p27 protein level was decreased in β1-7721 and α5β1-7721 cells. Lane 1, mock cells; lane 2, α5-7721; lane 3, β1-7721; lane 4, α5β1-7721. Each result represented at least 3 independent experiments.

Figure 8 The percentage of cells in S phase was increased in SMMC-7721 cells plated on poly-HEME-coated petri dishes. The parental SMMC-7721 cells were plated on poly-HEME-coated petri dishes, and cultured in normal medium for 24 h (A), 48 h (B) or 72 h (C), respectively, then collected and analysed by flow cytometry. The cell cycle pattern (C) was similar to that of β1-7721 or α5β1-7721 cells.

DISCUSSION
We examined the effects of overexpressed α5β1 or β1 on tumor cell proliferation, which provide the evidence that overexpression of α5β1 or β1 inhibits the proliferation of human hepatocellular carcinoma cell line SMMC-7721 and this inhibition may be related to the insufficient ligands for overexpressed β1 integrins. This finding also demonstrated that the inhibition of cell cycle was due to a specific growth arrest at S-phase that involved an increase in the protein level of p21cip1 and p27kip1.

This study showed that cyclin D1 expression was not affected by transfection events. So the early G1 phase
progression may not be influenced under this condition. However, the protein levels of p21<sup>α1</sup> and p27<sup>kip1</sup> were increased in β1 or α5β1 transfectant cells, which might be the major reason why S-phase delay occurred. It was previously reported that p21<sup>α1</sup> and p27<sup>kip1</sup> were assembly factors rather than inhibitors of cdk4/6 kinases<sup>30</sup>, but were inhibitors of cdk2 kinase, which is the key kinase contributing to G1/S transition and S phase progression. One CK1 protein, p21<sup>α1</sup>, the first cyclin-dependent kinase inhibitor to be identified<sup>31</sup>, has also a separate cdk2 binding site in its N-terminal region (amino acid 53-58) and optimal cyclin/cdk inhibition requires binding to this site as well as one of the cyclin binding domains. Furthermore, p21<sup>α1</sup> interacts with proteins such as PCNA, c-Myc and E2Fs that control DNA replication and other S phase events<sup>32</sup>. Meanwhile, evidences have shown that p27<sup>kip1</sup> plays a key role in the regulation of the proliferation of tumor cells in response to signals from ECM<sup>13</sup>. Therefore, increased p21<sup>α1</sup> and p27<sup>kip1</sup> proteins can induce S-phase delay in β1- and α5β1-transfected cells.

In many cell types, the intracellular concentration of p2<sup>α1</sup> is mainly controlled at the posttranscriptional level, and its degradation is initiated by phosphorylation with a target enzyme, cdk2<sup>33</sup> or other enzymes, such as PKB<sup>34</sup>, and completed by the ubiquitin-proteasome pathway. p21<sup>α1</sup> protein can also be phosphorylated by PKB<sup>35</sup>. In this study, we found that PKB level of phosphorylated form was decreased in β1- or α5β1- transfected cells, accompanied by the increase of p21<sup>α1</sup> and p27<sup>kip1</sup> to a certain extent. These findings indicate that the decrease of active form of PKB may interpret the accumulation of p21<sup>α1</sup> and p27<sup>kip1</sup> bona fide, which in turn, interferes with the cell cycle at S phase.

This study suggested that overexpression of β1 or α5β1 integrin in SMMC-7721 cells could induce S-phase delay. The mechanism underlying this phenomenon may be due to two kinds of possibilities. One is “integrin-mediated death” (IMD) described by Stupack and his colleagues<sup>34, 35</sup>, that is, an unligated integrin promotes apoptosis of cells. It is well known that α5β1 integrin, in general, acts as the effector protein of cell proliferation, such as endothelial cells in the vascular system<sup>36</sup>. In this study, however, we showed that overexpression of β1 or α5β1 induced S-phase delay. When cells were plated on FN/LN-coated culture dishes, they were subjected to attachment and spreading compared with the mocked and α5-7721 cells. Moreover, for the parental cells, they underwent S-phase delay and apoptosis when they were deprived of attachment by plating them on poly-HEME-coated petri dishes. Therefore, we postulated that the relative lack of ECM might be involved in S-phase delay triggered by overexpression of β1 or α5β1 integrin gene in SMMC-7721 cells. Another possibility is the trans-dominate integrin inhibition, which is defined as the occupancy of one integrin by its ligand, can inhibit the functions of other integrins<sup>37-40</sup>. For example, integrin α5β1 is essential for angiogenesis<sup>36</sup>, but α5β3 may suppress the functions of integrin α5β1, if β3 integrins prevail in the endothelial cells<sup>41, 42</sup>. It was reported that the basal integrin repertoire in hepatocellular carcinoma cells was characterized by the expression of several potential laminin receptors of the integrin family, such as α1β1, α2β1 and α6β1<sup>41, 42</sup>. So the overexpression of β1 may preferentially dimerize with α1, α2 and α6, the subunits of the receptors of laminin or collagen (they were lost in this in vitro model). Therefore, if these α subunits are occupied, the functions of integrin α5β1 may be suppressed, including its capacity to block cell cycle arrest.

ACKNOWLEDGEMENTS

We are grateful to Dr. Mara Brancaccio from the University of Torino (Torino, Italy), and Dr. Sue E. Craig from the University of Manchester (Manchester, UK) for their gifts of the plasmids. We also acknowledge the Manchester Medical Board (MB) in New York, U.S.A. for its kind support to our research.

REFERENCES

1 Lukashev ME, Werb Z. ECM signalling: orchestrating cell behaviour and misbehaviour. Trends Cell Biol 1998; 8: 437-441
2 Giancotti FG, Ruoslahti E. Integrin signaling. Science 1999; 285: 1028-1032
3 Assaadon RK. Anchorage-dependent cell cycle progression. J Cell Biochem 1997; 66: 136-141
4 Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 1994; 124: 619-626
5 Schwartz MA. Integrin signaling revisited. Trends Cell Biol 2001; 11: 466-470
6 Nicholson KM, Anderson NG. The protein kinase B/Akt signalling pathway in human malignancy. Cell Signal 2002; 14: 381-395
7 Sherr CJ, Roberts J.M. CDK inhibitors: positive and negative regulators of G1-phase progression. Gene Dev 1999; 13: 1503-1512
8 Giancotti FG, Mariero F. Integrin-mediated adhesion and signaling in tumorigenesis. Biochim Biophys Acta 1994; 1198: 47-64
9 Giancotti FG, Ruoslahti E. Elevated levels of the c51 fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. Cell 1990; 60: 849-859
10 Varner JA, Emerson DA, Juliano RL. Integrin c51 expression negatively regulates cell growth: reversal by attachment to fibronectin. Mol Biol Cell 1995; 6: 725-740
11 Hata GF, Ye F, Cao LH, Zha XL. Overexpression of integrin α5c1 in human hepatocellular carcinoma cell line suppresses cell proliferation in vitro and tumorigenicity in nude mice. Mol Cell Biochem 2000; 207: 49-55
12 Wang D, Sun L, Zborowska E, Willson JK, Gong J, Verrarraghavan J, Brattain MG. Control of type II transforming growth factor-β receptor expression by integrin ligation. J Biol Chem 1999; 274: 12840-12847
13 Henriot P, Zhong ZD, Brooks PC, Weinberg KI, DeClerck YA. Contact with fibrillar collagen inhibits melanoma cell proliferation by up-regulating p27<sup>Kip1</sup>, Proc Natl Acad Sci USA 2000; 97: 10026-10031
14 Su JM, Gui L, Zhou YP, Zha XL. Expression of focal adhesion kinase and α5 and β1 integrins in carcinomas and its clinical significance. World J Gastroenterol 2002; 8: 613-618
15 Munsterberg AE, Lassar AB. Combinatorial signals from the neural tube, floor plate and notochord induce myogenic bHLH gene expression in the somite. Development 1995; 121: 651-660
16 Takano Y, Kato Y, van Dieijen P, Masuda M, Itomii H, Okayasu I. Cyclin D2 overexpression and lack of p27 corolate positively and cyclin E inversely with a poor prognosis in gastric cancer cases. Am J Pathol 2000; 156: 585-594
17 Chen B, He L, Salvell VH, Jenkins JJ, Parham DM. Inhibition of the interferon-gamma/signal transducers and activators of transcription (STAT) pathway by hypermethylation at a STAT-binding site in the p21<sup>cip1</sup> promoter region. Cancer Res 2000; 60: 3290-3298
18 Kim J, Han I, Kim Y, Kim S, Oh ES. C-terminal heparin-binding domain of fibronectin regulates integrin-mediated cell spreading but not the activation of mitogen-activated protein kinase. Biochem 2001; 306: 239-245
19 Heino J, Ignatou RA, Hemler ME, Crouse C, Massague J. Regulation of cell adhesion receptors by transforming growth factor-β. Concomitant regulation of integrins that share a common β1 subunit. J Biol Chem 1989; 264: 380-388
20 Bellis SL, Newman F, Friedman EA. Steps in integrin β1-chain glycosylation mediated by TGFβ1 signaling through Ras. J Cell Physiol 1999; 181: 33-44
21 Yan Z, Chen M, Peruchu M, Friedman E. Oncogenic Ki-ras but not oncogenic Ha-ras blocks integrin β1-chain maturation in colon epithelial cells. J Biol Chem 1997; 272: 30298-30303
22 Henriot P, Alahari SK, Juliano RL. Integrin signaling and cell growth control. Curr Opin Cell Biol 1998; 10: 220-231
23 Sherr CJ, Roberts JM. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev 1995; 9: 1149-1163
24 Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21
Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 1993; 75: 805-816

Zhang Y, Rishi AK, Dawson M1, Tschang R, Farhana L, Boyanapalli M, Reichert U, Shroo B, Van Buren EC, Fontana JA. S-phase arrest and apoptosis induced in normal mammary epithelial cells by a novel retinoid. Cancer Res 2000; 60: 2025-2032

Shenberger JS, Dixon PS. Oxygen induces S-phase growth arrest and increases p53 and p21WAF1/CIP1 expression in human bronchial smooth-muscle cells. Am J Respir Cell Mol Biol 1999; 21: 395-402

Brazil DP, Hemmings BA. Ten years of protein kinase B signalling: a hard Akt to follow. Trends Biochem Sci 2001; 26: 657-664

Delcommenne M, Tan C, Gray V, Rue L, Woodgett J, Dedhar S. Phosphoinositide-3-OH kinase-dependent regulation of glycolysis synthase kinase 3 and protein kinase B/AKT by the integrin-linked kinase. Proc Natl Acad Sci USA 1998; 95: 11211-11216

Persad S, Attwell S, Gray V, Mawji N, Deng JT, Leung D, Yan J, Sanghera J, Walsh MP, Dedhar S. Regulation of protein kinase B/Akt-serine 473 phosphorylation by integrin-linked kinase: critical roles for kinase activity and amino acids arginine 211 and serine 343. J Biol Chem 2001; 276: 27462-27469

Sherr CJ. The Pecozzeller lecture cancer cell cycles revisited. Cancer Res 2000; 60: 3689-3695

el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Morer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. Cell 1993; 75: 817-825

Dotto GP. p21^{WAF1/CIP1}, more than a break to the cell cycle? Biochim Biophys Acta 2000; 1471: M43-M56

Elledge SJ, Harper JW. The role of protein stability in the cell cycle and cancer. Biochim Biophys Acta 1998; 1377: M61-M70

Stupack DG, Puente XS, Boutsaboualoy S, Storgard CM, Cheresh DA. Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. J Cell Biol 2001; 155: 459-470

Cheresh DA, Stupack DG. Integrin-mediated death: an explanation of the integrin-knockout phenotype? Nat Med 2002; 8: 193-194

Yang JT, Rayburn H, Hynes RO. Embryonic mesodermal defects in α5 integrin-deficient mice. Development 1993; 113: 1093-1105

Schwartz MA, Ginsberg MH. Networks and crosstalk: integrin signalling spreads. Nat Cell Biol 2002; 4: E65-E68

Blystone SD, Graham IL, Lindberg FP, Brown EJ. Integrin αvβ3 differentially regulates adhesive and phagocytic functions of the fibronectin receptor α5β1. J Cell Biol 1994; 127: 1129-1137

Diaz-Gonzalez F, Forsyth J, Steiner B, Ginsberg MH. Trans-dominant inhibition of Integrin function. Mol Biol Cell 1996; 7: 1939-1953

Blystone SD, Slater SE, Williams MP, Crow MT, Brown EJ. A molecular mechanism of integrin crosstalk: αvβ3 suppression of calcium/ calmodulin-dependent protein kinase II regulates α5β1 function. J Cell Biol 1999; 145: 889-897

Simon KO, Nudd EM, Abraham DG, Rodan GA, Duong LT. The αvβ3 integrin regulates α5β1-mediated cell migration toward fibronectin. J Biol Chem 1997; 272: 29380-29389

Volpes R, van den Oord JJ, Desmet VJ. Integrins as differential cell lineage markers of primary liver tumors. Am J Pathol 1993; 142: 1483-1492

Scoazec JY, Fléjou JF, D’Errico A, Fiorentino M, Zamparelli A, Bringuier AF, Feldmann G, Grigioni WF. Fibrolamellar carcinoma of the liver: composition of the extracellular matrix and expression of cell-matrix a cell-cell adhesion molecules. Hepatology 1996; 24: 1128-1136

Torimura T, Uneo T, Kin M, Inuzuka S, Sugawara H, Tamaki S, Tsujiri R, Sujaku K, Sata M, Tanikawa K. Coordinated expression of integrin αvβ6 and laminin in hepatocellular carcinoma. Hum Pathol 1997; 28: 1131-1138

Ozaki I, Yamamoto K, Mizuta T, Kajihara S, Fukushima N, Setoguchi Y, Morito F, Sakai T. Differential expression of laminin receptors in human hepatocellular carcinoma. Gut 1998; 43: 837-842

Masamoto A, Arai S, Otsuki M. Role of β1 integrins in adhesion and invasion of hepatocellular carcinoma cells. Hepatology 1999; 29: 68-74

Nejjar M, Hafdi Z, Dumortier J, Bringuier AF, Feldmann G, Scoazec JY. αvβ1 integrin expression in hepatocarcinoma cells: regulation and role in cell adhesion and migration. Int J Cancer 1999; 83: 518-525

Edited by Zhang JZ and Wang XL