CMLS, Cell. Mol. Life Sci. 61 (2004) 2071–2082
1420-682X/04/162071-12
DOI 10.1007/s00018-004-4165-1

CMLS Cellular and Molecular Life Sciences

Research Article

β_2-integrins mediate a novel form of chemoresistance in cycloheximide-induced U937 apoptosis

R.-C. Wu [a, b], Z. Wang [a, b, *], M.-J. Liu [a, b], D.-F. Chen [a, b] and X.-S. Yue [a, b]

[a] Department of Biological Sciences and Biotechnology, Tsinghua University, Beijing (China),
[b] Institute of Biomedicine, Tsinghua University, Beijing (China), Fax: +86 10 62772240,
e-mail: zwang@tsinghua.edu.cn

Received 15 April 2004; received after revision 18 May 2004; accepted 21 June 2004

Abstract. In this study with cycloheximide (CHX, an inhibitor of protein synthesis) and the human leukaemic cell line U937, a novel form of chemoresistance, which we termed sudden drug resistance (SDR), was identified using Hoechst33258 staining, Western blott and DNA Ladder. CHX^{high} (10–100 μg/ml)-induced apoptosis can spontaneously subside after 4–6 h or can be inhibited by short-term preincubation with CHX^{low} (2.5 μg/ml). Unlike typical multidrug resistance, SDR is not caused by reduced drug accumulation or altered protein expression, and may be associated with a non-P-glycoprotein mechanism. To uncover this underlying mechanism, we focused on U937 cell aggregation promoted by CHX, because cell adhesion has been suggested to influence cell survival and prevent apoptosis. EDTA, or anti-CD18 monoclonal antibody, but not EGTA, acetylsalicylic acid or RGDS tetrapeptide, abrogated this homotypic aggregation and greatly increased CHX-induced apoptosis in a time-dependent manner, while fibrinogen and soluble intercellular adhesion molecule-1 exerted opposite effects. These results establish that β_2-integrin engagement is a key mediator of SDR, although it may be non-exclusive. This finding supplements the classical basis of chemoresistance and may provide another opportunity for improved leukemia therapy.

Key words. Apoptosis; cycloheximide; U937 cell; β_2-integrin; drug resistance; PI-3K.

Apoptosis is a highly regulated cell death process characterized by a series of stereotypic morphological and biochemical features, including cell shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation and a proteolytic cleavage cascade by caspases [1, 2]. It plays a crucial role both during normal development and under certain pathological conditions in metazoans [3]. Evasion of apoptosis was recently listed as an essential hallmark of cancer [4], while the notion that apoptosis might influence the malignant phenotype can be traced back to the early 1970s [5]. In the last decade in particular, emerging knowledge of the molecular links between tumorigenesis, apoptosis and drug resistance has provided the main foundation for chemotherapeutic tumour eradication [6], though numerous problems remain to be resolved.

Cycloheximide (CHX) is a protein synthesis inhibitor that has been widely used in studies of apoptosis. In the human leukaemic cell line U937, it is known to be a mild apoptotic stimulant [7, 8]. However, in the early stage (within 12 h), which had been neglected previously, we have further defined a novel form of chemoresistance in which CHX^{high} (10–100 μg/ml)-induced apoptosis can spontaneously subside after 4–6 h and be inhibited by short-term preincubation with CHX^{low} (2.5 μg/ml). We termed this chemoresistance 'sudden drug resistance' (SDR) to distinguish it from multidrug resistance (MDR), which is generally induced by chronic drug ex-

* Corresponding author.

posure and related to a P-glycoprotein (P-gp) mechanism [9, 10].

Various findings are revealing alternative mechanisms for the anti-apoptotic actions of CHX. Concentrations of CHX that cause a modest and/or transient decrease in overall protein synthesis may prevent cell death by inducing expression of cytoprotective gene products such as Bcl-2, c-Fos and c-Jun, whereas higher concentrations of CHX may prevent cell death by suppressing production of presumptive killer genes [11–14]. But the ability of CHX to induce apoptosis concurrently with inhibition of protein synthesis within a short time implies that a new pattern should be accepted for the regulation of SDR. In our experimental system in particular, CHX also appears to promote cell adhesion that is primarily mediated by integrins, a large family of cell surface receptors, which have been suggested to control the apoptotic response [15]. In normal epithelial and endothelial cells, signalling by appropriately ligated integrins suppresses anoikis [16, 17] and even Fas-mediated apoptosis [18]. Recently, unligated integrins were also reported to induce apoptosis by recruitment of caspase-8 [19]. Although initial interest in this field may be rooted in cell positioning and neoplasia [20], cell-adhesion-mediated drug resistance (CAM-DR) proposed by Damiano et al. [21] to explain how some cells survive initial drug exposure directly associates the integrins with drug resistance. Hence, we speculated that a CAM-DR-like mechanism might be responsible for the regulation of SDR triggered by CHX.

All integrins are non-covalent $\alpha\beta$ heterodimers, each of which has its own binding specificity, signalling properties and restriction to certain cell types. According to their β chains, integrins are commonly divided into several subfamilies [22]. The leukocyte-specific subfamily named β_2-integrins consists of four different members: $\alpha_L\beta_2$ (CD11a/CD18, LFA-1), $\alpha_M\beta_2$ (CD11b/CD18, Mac-1), $\alpha_X\beta_2$ (CD11c/CD18, p150,95) and $\alpha_D\beta_2$ (CD11d/CD18). After activation, these leukocyte receptors can mediate cell-cell contacts by binding to intercellular adhesion molecules 1, 2, 3 and 4 (ICAM-1, -2, -3 and -4) without the involvement of any additional extracellular matrix (ECM) proteins [23]. Functionally, LFA-1/ICAM-1 has also been shown to prevent apoptosis in germinal centre B cells, cooperating with very late appearing antigen-4 (VLA-4)/vascular cell adhesion molecule-1 (VCAM-1) [24]. Thus, we took advantage of this opportunity to test a potential role for β_2-integrins in SDR, and found that abrogating homotypic aggregation of U937 cells in various ways was accompanied by the circumvention of SDR, whereas preincubation with the plated ligands of β_2-integrins such as sICAM-1 and fibrinogen (FB) greatly facilitated SDR. These results suggest that β_2-integrin-mediated cell adhesion is indeed a key mediator of SDR. Moreover, fibronectin (FN) slightly inhibited CHX-induced U937 apoptosis, further indicating the possibility of cooperative signalling by other integrins such as VLA-4.

Materials and methods

Cell culture

U937 cells were kindly provided by Dr Y.-H. Chen (Molecular Immunology Laboratory, Tsinghua University, Beijing, P. R. China), and grown in RPMI-1640 medium (GIBCO-BRL Life Technologies, Carlsbad, Calif.) supplemented with 10% standard fetal bovine serum (Hyclone Laboratories, Logan, Utah), 2 g/l sodium bicarbonate, 100 µg/ml streptomycin and 100 U/ml penicillin. The cultures were maintained at 37 °C in a humidified 5% CO_2 atmosphere.

Drug treatments

The following reagents, unless indicated otherwise, were all purchased from Sigma-Aldrich (Loius, Mo.). Etoposide (VP-16), CHX, wortmannin (WM), GF109203X and BOC-Asp(OMe)-fluoromethyl ketone (BOC-D.fmk; Enzyme Systems Products, Livermore, Calif.) were dissolved in dimethyl sulfoxide (DMSO). EDTA and EGTA were prepared in sterile water and the pH was adjusted to 7.4. Arg-Gly-Asp-Ser (RGDS tetrapeptide), acetylsalicylic acid (ASA), mouse IgG (Academy of Military Medical Sciences, Beijing, China) and anti-CD18 monoclonal antibody (mAb) (clone 68 5A5; NeoMarkers, Fremont, Calif.) were prepared in phosphate-buffered saline (PBS) and stored at –20 °C. Recombinant soluble human ICAM-1 (R&D Systems; Minneapolis, Minn.), FB and FN (from human plasma) were diluted in pyrogen-free water, and freshly plated to the wells as described below. In all experiments, exponentially growing cells were seeded at an initial cell concentration of 2×10^5/ml for the following 12 h of culture and exposed to treatment for the times indicated.

Assessment of apoptosis (Hoechst33258 staining)

After treatment, cell pellets were fixed in methanol, and then stained with 20 µg/ml Hoechst33258 solution. Following two washes with ice-cold PBS, they were observed under a fluorescence microscope (Leica DMLB and MPS60; Leica Microsystems, Wetzlar GmbH, Wetzlar, Germany). Apoptotic cells were identified based on the fragmented nuclear morphology and recorded in a continuous visual field (total number of cells >600). The percentage of apoptosis was calculated using the formula: 100 × (number of apoptotic cells/total number of cells).

Western blot

Cells were harvested and washed twice with ice-cold PBS. The lysates were obtained with TEN-T buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 5 mM EDTA pH 8.0, 1% Triton X-100, 1 mM PMSF, 2 µg/ml aprotinin), and

then subjected to 10,000 g centrifugation at 4 °C for 20 min. Protein levels were quantified using a bicinchoninic acid assay (Beyotime Biotechnology, Haimen, China). Total proteins 50 µg/lane were fractioned by 7.5% for MDR1 (clone JSB-1; Zymed Laboratories, South San Francisco, Calif.), 12.5% for c-Fos (clone 4), c-Jun (clone H-79), Bcl-x_L (clone H-5), Bax (clone N-20) (Santa Cruz Biotechnology, Santa Cruz, Calif.), Bcl-2 (lot M051096; BD Biosciences Clontech, San Jose, Calif.) and β-actin (clone AC-74; Sigma-Aldrich) and 15% for caspase-3 (clone H-277; Santa Cruz Biotechnology) SDS-polyacrylamide gels electrophoresis, and subsequently transferred to PVDF sheets. After blocking with TTBS (50 mM Tris-HCl pH 7.4, 0.5 M NaCl, 0.05% Tween 20) containing 5% (w/v) skim milk, the sheets were incubated with first antibodies at 4 °C overnight. Blots were then detected with an ECF Western blotting kit. The densities of sample bands were determined using a fluorescence scanner (Storm 860) and analysed with ImageQuant software (Amersham Biosciences, Amersham, UK).

DNA fragmentation analysis (DNA ladder)

At the end of treatment, cells (1×10^6) were harvested and lysed in 100 µl buffer (10 mM Tris-HCl pH 7.4, 10 mM EDTA pH 8.0, 0.5% Triton X-100). The supernatant was obtained by centrifugation at 14,000 g for 10 min, and then incubated with RNase A (400 µg/ml) at 37 °C for 60 min. After proteins had been removed by incubation with proteinase K (200 µg/ml) at 50 °C for 30 min, 20 µl 5 M NaCl and 120 µl isopropanol were added to the lysate which was stored at –20 °C overnight. The precipitated DNA pellets were then dissolved in Tris-acetate-EDTA buffer, electrophoresed in a 1.5% agarose gel, stained with ethidium bromide (EB), and photographed under UV illumination.

Total RNA extraction and RT-PCR analysis

Total RNA was isolated from treated cells using the TRIzol reagent (Sangon, Shanghai, P. R. China) and immediately reverse-transcribed using oligo-p$(dT)_{18}$ primer and M-MuLV reverse transcriptase (Sangon) according to the manufacturer's protocol. In the same cDNA preparation, *c-fos*, *c-jun*, *bcl-2*, *bcl-x_L* and *bax* were amplified by PCR using *β-actin* as internal standard. The sequences of the oligonucleotide primers used for amplification were for *c-fos* sense 5′-GCA GAC TAC GAG GCG TCA TC-3′ and anti-sense 5′-TTC AGC AGG TTG GCA ATC TC-3′, defining a 542-bp product; for *c-jun* sense 5′-ATG ACT GCA AAG ATG GAA ACG ACC-3′ and anti-sense 5′-GAT GTG CCG TTG CTG GAC TGG AT-3′, defining a 264-bp product; for *bcl-2* sense 5′-TGG AGG AGC TCT TCA GGG AC-3′ and anti-sense 5′-CAC TGA CCC CAC CGA ACT CA-3′, defining a 72-bp product; for *bcl-x_L* sense 5′-GCG TAG ACA AGG AGA TGC AGG T-3′ and anti-sense 5′-GGT CAT TCA GGT AAG TGG CCA T-3′, defining a 72-bp product; for *bax* sense 5′-CCC AAG ACC AGG GTG GTT G-3′ and anti-sense 5′-AAA GAT GGT CAC GGT CTG CC-3′, defining a 74-bp product; and for *β-actin* sense 5′-TGG ACT TCG AGC AAG AGA TGG-3′ and anti-sense 5′-ATC TCC TTC TGC ATC CTG TCG-3′, defining a 289-bp product. A 25-µl PCR reaction mixture contained 5 µl cDNA, 1 × PCR buffer, 2 µM $MgCl_2$, 0.2 mM dNTP, 0.5 µM of each primer, and 1.25 U of Taq DNA polymerase (Sangon). After an initial denaturation step at 94 °C for 5 min, the PCR profile for *c-fos* amplification with 30 cycles and for *bcl-2*, *bcl-x_L*, *bax* and *β-actin* amplification with 23 cycles was 94 °C, 1 min; 62 °C, 45 s; 72 °C, 45 s. For *c-jun* amplification with 23 cycles, it was 94 °C, 1 min; 65 °C, 45 s; 72 °C, 45 s. A final extension was performed at 72 °C for 8 min. Then the PCR products were separated on 1.5% agarose gels, stained with EB, and photographed under UV illumination.

Preparation of sICAM-1-, FB- and FN-coated wells

FB (0.3 ml of 500 µg) diluted in pyrogen-free water was dispensed in 2-cm^2 24-well plates (Costar, Cambridge, Mass.), and allowed to dry at 37 °C [25]. sICAM-1 (6 µg/well) and FN (15 µg/well) were coated on 24-well plates by overnight incubation at 4 °C [26], and then allowed to dry in a vacuum desiccator. The amounts used for coating were chosen to give the maximum cell binding.

Flow cytometric analysis of CD18

Cells treated with or without CHX were collected by centrifugation and washed with PBS. For extracellular staining, they were then incubated with 10 µg/ml mouse IgG (negative control) or anti-CD18 mAb (68 5A5) at 4 °C for 30 min, washed twice with ice-cold PBS, and resuspended in 200 µl PBS containing 15 µg/ml fluorescein isothiocyanate (FITC)-conjugated horse anti-mouse IgG (Vector Laboratories, Burlingame, Calif.) at 4 °C for another 30 min. Finally, fluorescence measurement was carried out on a FACSCalibur fluorescence-activated cell sorter using the CELLQuest software (Becton Dickinson, Franklin Lakes, N. J.).

Results

CHX-induced apoptosis of U937 cells is dose- and caspase-dependent

U937 cells treated with CHX expressed the typical changes characteristic of apoptosis: nuclear condensation and fragmentation (fig. 1A, right panel) in a dose-dependent manner (fig. 1A, left panel). In particular, the cell-permeable, pan-caspase inhibitor, BOC-D.fmk, at 20 µM completely blocked these changes (fig. 1A, left panel), further suggesting the indispensable involvement of caspases in CHX-induced U937 apoptosis.

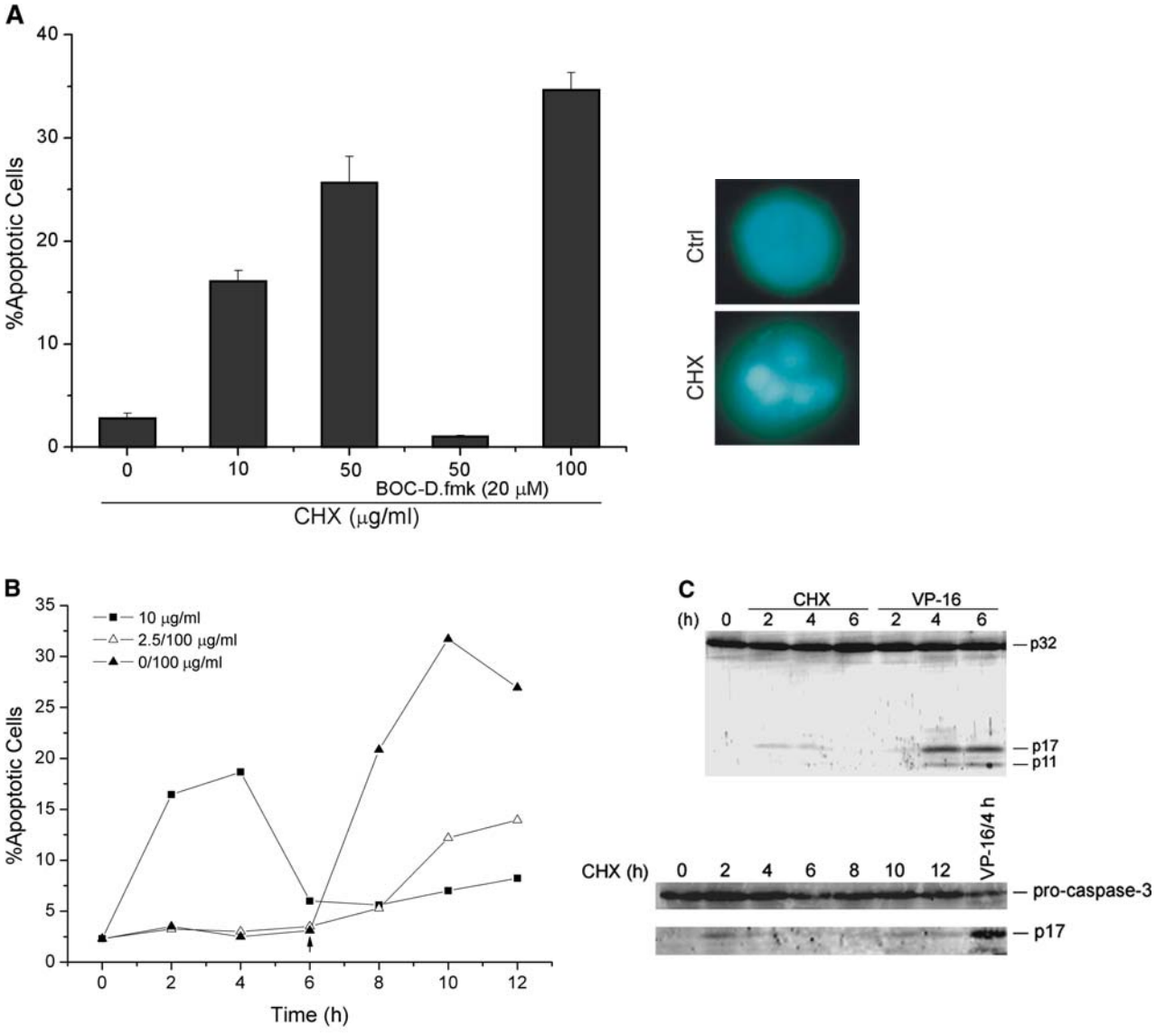

Figure 1. Unprompted and self-imposed inhibition of CHX-induced U937 apoptosis. First, nuclear morphologic changes of U937 cells treated with a drug vehicle (DMSO, Ctrl) or CHX (10 μg/ml) for 4 h were identified using Hoechst33258 staining (*A*, right panel). Then, U937 cells were incubated with different concentrations of CHX for 4 h in the presence or absence of BOC-D.fmk (20 μM). Apoptosis was assessed by Hoechst33258 staining (*A*, left panel). Results are expressed as the means ± SD of three independent experiments. Subsequently, the time-dependent manner of CHX (10 μg/ml) and VP-16 (50 μM)-induced apoptosis was assessed using Hoechst33258 staining (*B*), and the cleavage of procaspase-3 was analysed by Western blot (*C*). Finally, higher concentrations of CHX (100 μg/ml) were also employed to trigger apoptosis in U937 cells pretreated with a drug vehicle (0/100 μg/ml) or 2.5 μg/ml CHX (2.5/100 μg/ml) for 6 h (arrow). At the indicated time points, apoptosis was assessed by Hoechst33258 staining (*B*) and DNA ladder (*D*). Results are representative of two independent experiments.

Aberrant kinetics of CHX-induced U937 apoptosis

However, CHX (10 µg/ml)-induced U937 apoptosis was time-independent and encountered an unprompted inhibition after 4 h (fig. 1B). To confirm these aberrant kinetics, we also examined caspase-3 activation, as judged by the appearance of the 17-kDa subunit (p17), a major cleaved product of the 32-kDa dormant zymogen [27]. As expected, the processing of caspase-3 was detectable only within 2–4 h but not at other time points of CHX treatment (fig. 1C), whereas a time-dependent cleavage, which started to occur within 2 h and continued to sharply increase thereafter, was triggered by VP-16 (50 µM) (fig. 1C). These data suggested that an idiographic pattern might be applied to CHX-induced U937 apoptosis.

To explain the unprompted inhibition, we hypothesized that following incubation with CHX, a form of acquired drug resistance might be rapidly conferred to U937 cells. Supporting this, pretreatment with a relatively low concentration of CHX (2.5 µg/ml) for 6 h in comparison with a drug vehicle (DMSO) was shown to drastically impair the apoptotic effects of a subsequently higher concentration of CHX (100 µg/ml), as assessed by Hoechst33258 staining (fig. 1B) and DNA ladder (fig. 1D), another marker of apoptosis related to extensive DNA digestion. At the same time, both kinds of preincubation displayed noncytotoxicity regardless of the distinct sensitivities for the subsequent insults (fig. 1B), excluding the possibility that the unprompted and self-imposed inhibition could be attributed to the individual diversity of survival thresholds or intrinsic resistance. Historically, MDR has commonly been associated with several rounds of drug exposure or chemotherapy, and occurred slowly. Therefore, to distinguish the new phenomenon, we have proposed the term 'sudden drug resistance' (SDR).

Cell adhesion, rather than specific gene expression, is coupled to SDR

To investigate whether expression of oncogenes (*c-fos* and *c-jun*) and the *bcl-2* family members known to suppress (*bcl-2*, *bcl-x_L*) or promote (*bax*) apoptosis were altered in CHX-treated cells, RT-PCR was performed on total RNA collected from five different cell samples. As shown in figure 2A, only *c-fos* was transiently activated at 2 h of CHX treatment, while *bax* was slightly suppressed with the same timing. But removal of CHX at 4 h (lane 5) showed no impact on their transcription, suggesting that the early responses might be merely an acclimation behaviour of cells towards deleterious environments. In fact, nearly unaltered protein levels of all these genes (fig. 2B), in concert with full inhibition of translation by CHX (10 µg/ml) [28], also supported the notion that the short-lived transcripts might lack apoptotic relevance and ruled out the possibility that specific gene expression was coupled to SDR. Similarly, the equally low expression of MDR1 or P-gp (fig. 2B), a 170-kDa multidrug efflux pump, which is also inherently expressed in normal monocytic lineages but fails to show P-gp function examined by measuring verapamil-sensitive dye retention [29], indicated that SDR might be not regulated by a P-gp mechanism, either.

Treatment with CHX also induced homotypic aggregation of U937 cells (fig. 3A). This process was completely blocked by EDTA but not EGTA pretreatment (fig. 3A), because bound Mg^{2+}, rather than bound Ca^{2+}, plays a positive regulatory role in the control of leukocyte integrin functions [30, 31]. To identify if this homotypic aggregation was coupled to SDR, we then evaluated the extent of apoptosis under corresponding conditions using Hoechst 33258 staining and a DNA ladder (fig. 3B). As expected, pretreatment with EDTA greatly increased CHX- but not VP-16-induced apoptosis, while EGTA had no effect (fig. 3B). In particular, these functions of EDTA were shown to be reversible when given enough time (< 4 h) to re-bind divalent cations such as Mg^{2+} from fresh medium (fig. 3C). This result not only strengthened the possibility that EDTA functioned through the chelation of Mg^{2+}, but also implied that an absence of EDTA removal might be more suitable for the time-response experiments. However, the

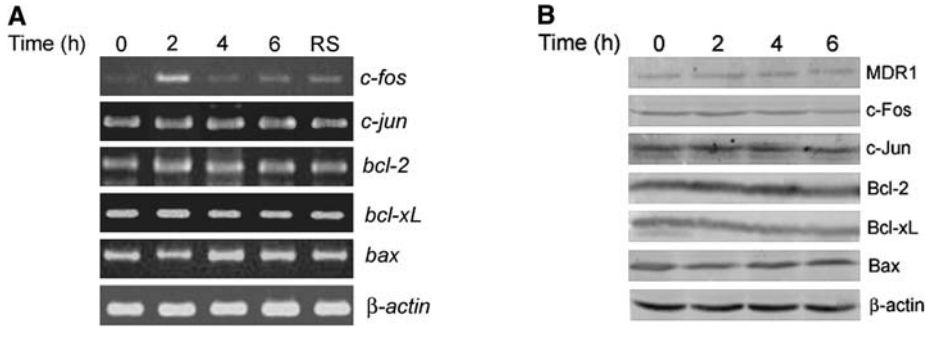

Figure 2. Effect of CHX on specific gene expression. U937 cells were cultured with CHX (10 µg/ml). After 0, 2, 4 and 6 h, total RNA was isolated, and *c-fos*, *c-jun*, *bcl-2*, *bcl-x_L*, *bax* and *β-actin* mRNAs were amplified by RT-PCR (*A*). As an exception, total RNA in lane 5 (RS) was prepared from a special sample resuspended in fresh medium for 2 h after treatment with CHX (10 µg/ml) for 4 h. Whole-cell lysates were also blotted for MDR1 and the proteins encoded by the above six genes (*B*).

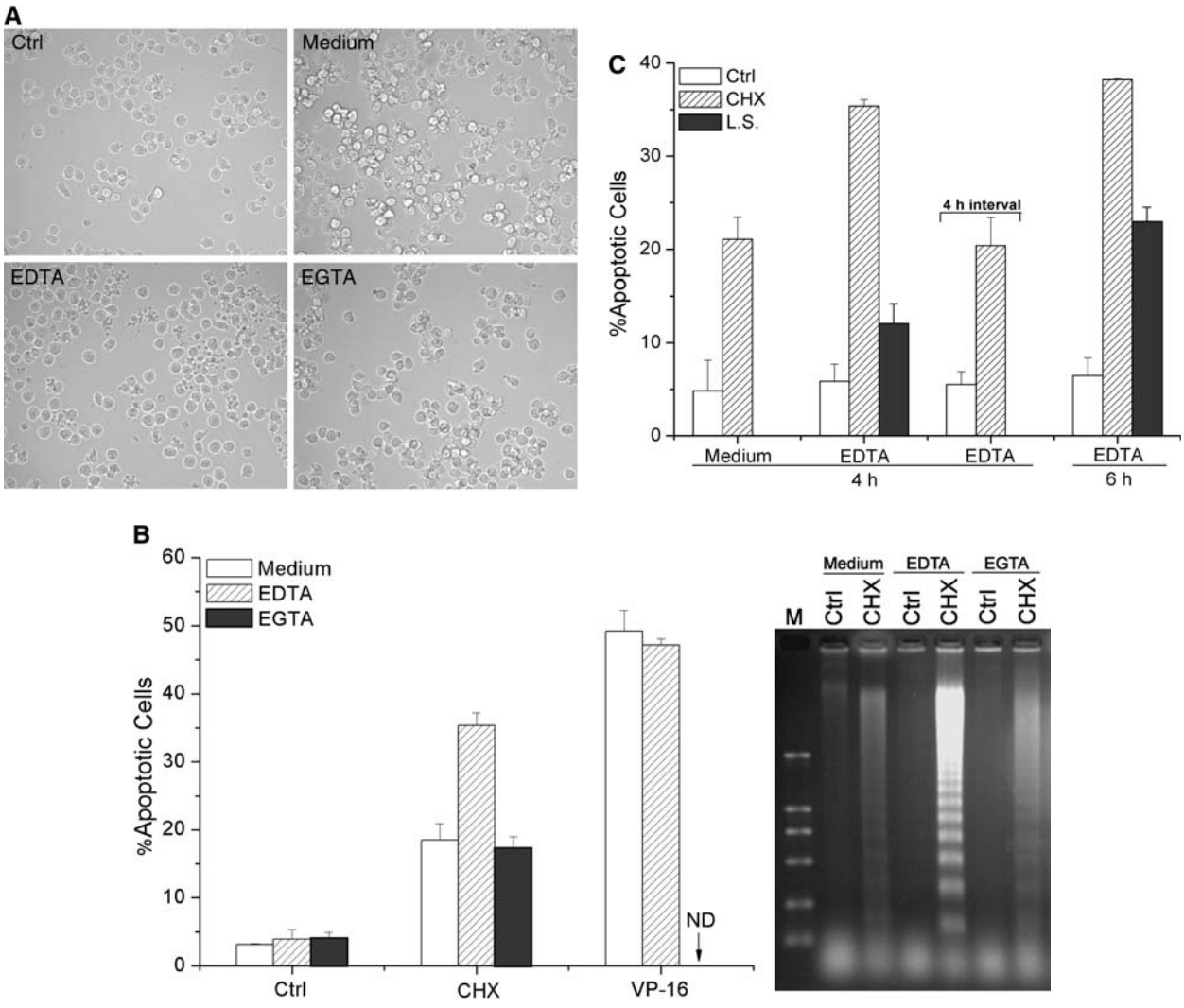

Figure 3. EDTA blocks U937 homotypic aggregation promoted by CHX and markedly increases apoptosis. U937 cells were pretreated with medium, EDTA (5 mM) or EGTA (5 mM), for 90 min, and then, after resuspension in fresh medium, a drug vehicle (Ctrl), CHX (10 μg/ml), or VP-16 (50 μM) were added immediately (*A*, *B*) or after an interval of 4 h for the following 4 (*A*–*C*) or 6 h of culture. Digital images were taken at × 200 magnification (Leica DMIRB, DC Viewer V3.2.0) (*A*). Long-term incubation of the cells with EDTA (5 mM) was also performed (L.S.) (*C*). Apoptosis was assessed by Hoechst33258 staining (*B*, left panel and *C*) and DNA ladder (*B*, right panel). Results are expressed as the means ± SD of three independent experiments. ND, not detected.

resultant cytotoxicity (fig. 3C, L.S.) and other uncertain effects of long-term (>4 h) incubation with EDTA in fact hampered such studies. Accordingly, we could not acquire satisfying data of the time-dependent impact of EDTA on CHX-induced apoptosis (fig. 3C), but the above results per se adequately established the fundamental role of cell adhesion in SDR.

Serum withdrawal confers an increased sensitivity to CHX-induced apoptosis, but maintains SDR

To simplify the experimental system, we next performed an assay to test if serum was necessary for SDR. As shown in figure 4A, serum withdrawal conferred an increased sensitivity to apoptosis induced by CHX, but still retained unprompted inhibition (compare with fig. 2B), indicating that SDR could take place independently of serum, but certain elements in serum such as growth factors and ECM proteins might cooperate in regulating CHX-induced apoptosis of U937 cells. In addition, homotypic aggregation was also observed at 4 h, although it was weaker (fig. 4B). Based on these findings, we then focused on β_2-integrins that are thought to mediate cell-cell adhesion without the involvement of additional ECM proteins.

β_2-integrin engagement is a key mediator of SDR

First, we examined the effects of inhibitors for cell adhesion mediated by various integrins on CHX-induced

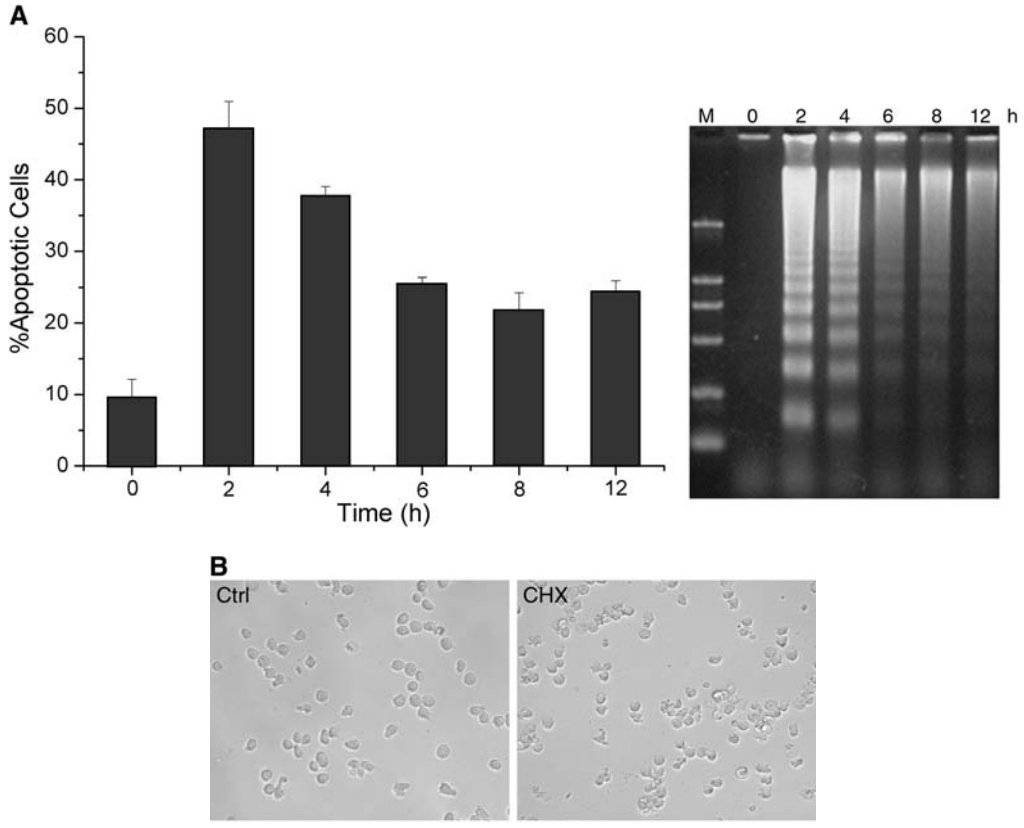

Figure 4. Serum withdrawal confers an increased sensitivity to apoptosis induction by CHX, but maintains SDR. U937 cells were suspended in serum-free medium and then treated with 10 µg/ml CHX for the indicated times. Apoptosis was assessed by Hoechst33258 staining (*A*, left panel) and DNA ladder (*A*, right panel). Results are expressed as the means ± SD of three independent experiments. At 4 h of treatment, with a drug vehicle as control (Ctrl), digital images were taken at × 200 magnification (*B*).

apoptosis. When monoclonal antibody (68 5A5) directed to CD18 at 100 µg/ml was used to pretreat U937 cells, it noticeably promoted CHX-induced apoptosis under serum-free conditions, while mouse IgG was without effect (fig. 5A), as assessed by Hoechst33258 staining and DNA ladder and in comparison to no pretreatment (None). mAb 68 5A5, not mouse IgG, also abrogated homotypic aggregation at the same time (fig. 5B), and even circumvented SDR (fig. 5C). In contrast, RGDS, a tetrapeptide known to disrupt integrin-ECM binding and $\alpha_{IIb}\beta_3$-mediated platelet aggregation [22, 32], was found to strongly inhibit CHX-induced apoptosis, whilst ASA, an anti-thrombotic agent, showed no significant impact (fig. 5A). These results suggested that β_2-integrin engagement was indeed a powerful mediator of SDR.

To further confirm this potential involvement of β_2-integrin engagement, counterpart ligands such as sICAM-1 and FB [31] were then employed to facilitate SDR. As expected, preincubation with plated sICAM-1 or FB dramatically inhibited CHX-induced apoptosis under serum-free conditions (fig. 5D). Furthermore, LFA-1/ICAM-1 has been suggested to cooperate with VLA-4/VCAM-1 in inhibiting apoptosis of germinal centre B cells [24], and we therefore examined whether VLA-4 was also associated with SDR. Given that VCAM-1 was not expressed on U937 cells, we instead examined the effect of FN, which is another ligand of VLA-4 [31] and largely exists in serum. As shown in figure 5D, FN also inhibited CHX-induced apoptosis slightly. This result further indicated the possibility of cooperative signalling by non-β_2-integrins such as VLA-4.

Surface density of CD18 is not altered by CHX treatment

Fluorescence-activated cytofluorometric analysis using anti-CD18 mAb (68 5A5) revealed no increase in the surface density of CD18 after CHX treatment of U937 cells (fig. 6). This equivalent binding suggested that the functional activation of β_2-integrins by CHX might result from conformational changes.

Active phosphatidylinositol 3-kinase, but not protein kinase C, is required for SDR

To simply explore whether PI-3K or PKC are involved in the signalling pathways of SDR, U937 cells were incubated with the phosphatidyl 3-kinase (PI-3K) inhibitor WM and protein kinase C (PKC) inhibitor GF109203X for 30 min prior to the addition of CHX. Preincubation

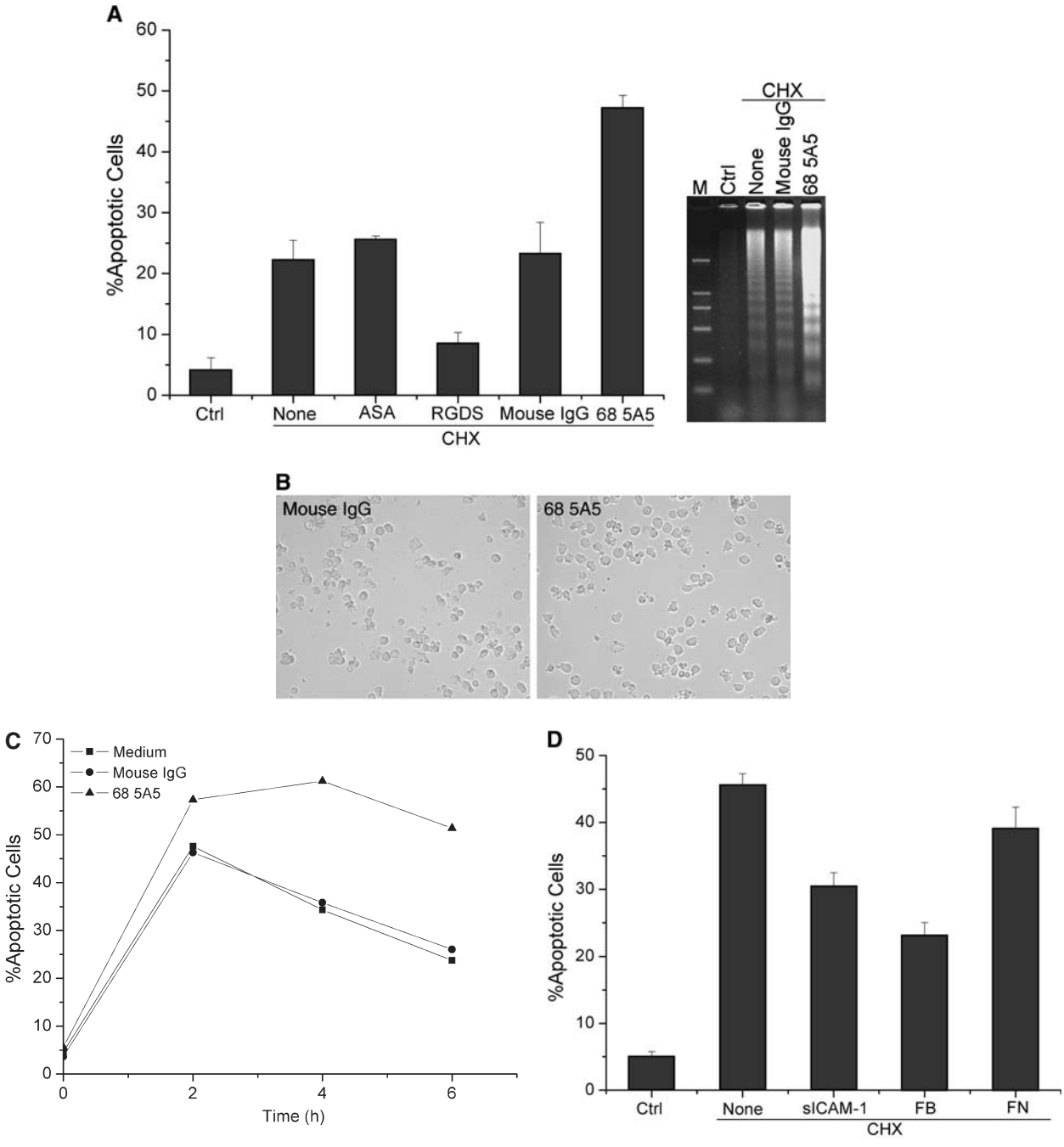

Figure 5. β_2-integrins are involved in the regulation of SDR. Under serum-free conditions, U937 cells were either untreated (None) or pretreated with ASA (1 mM), RGDS (2 mM), mouse IgG (100 µg/ml) or anti-CD18 mAb (68 5A5, 100 µg/ml) for 60 min, and then CHX (10 µg/ml) was added for the following 6 h (*A*, *B*) or the indicated times (*C*) of culture. Apoptosis was assessed by Hoechst33258 staining and DNA ladder. Cells treated with a drug vehicle (Ctrl) served as control. At 4 h of treatment, digital images were taken at × 200 magnification (*B*). Then, FB, sICAM-1 and FN were allowed to adhere in 24-well plates as described in Materials and methods. U937 cells were cultured for 60 min on uncoated (None) or coated wells under serum-free conditions, and then CHX (10 µg/ml) was added for the following 2 h of culture (*D*). Apoptosis was assessed by Hoechst33258 staining. Cells treated with a drug vehicle (Ctrl) served as control. Results are expressed as the means ± SD of three independent experiments.

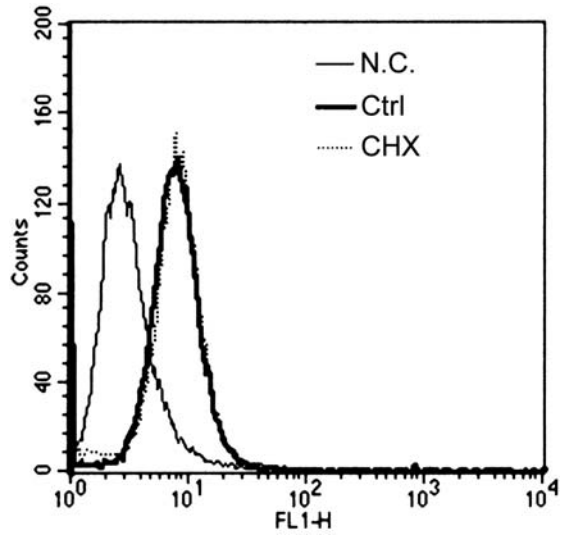

Figure 6. Effect of CHX on surface density of CD18. Flow cytometry of U937 cells with anti-CD18 mAb (68 5A5) was used to show the surface distribution of CD18 before and after incubation of the cells with CHX (10 µg/ml) for 4 h. Mouse IgG served as negative control (N.C.).

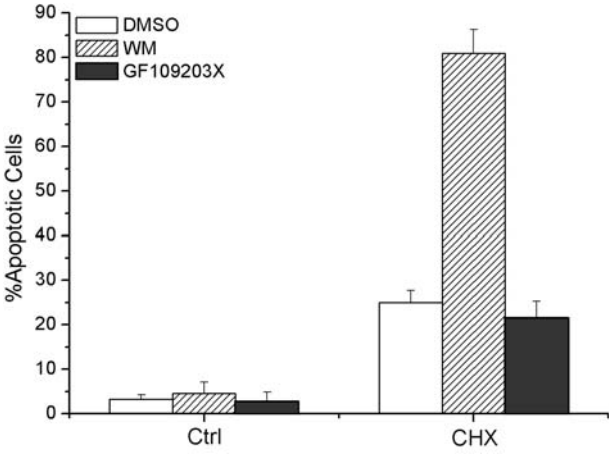

Figure 7. CHX effect is highly potentiated by WM but unaffected by GF109203X. U937 cells were pretreated with DMSO, WM (0.5 µM) and GF109203X (1 µM) for 30 min, and then a drug vehicle (Ctrl) or CHX (50 µg/ml) were added for the following 4 h of culture. Apoptosis was assessed by Hoechst33258 staining. Results are expressed as the means ± SD of three independent experiments.

with WM resulted in an over twofold increase in CHX-induced apoptosis, whilst GF109203X had no effect (fig. 7). But the same concentration of GF109203X has been demonstrated to strongly inhibit VP-16-induced U937 apoptosis (data not shown). These results suggested that PI-3K rather than PKC might participate in the regulation of SDR.

Discussion

CHX, long recognized for its ability to inhibit protein synthesis, has been known to stimulate or to prevent apoptosis according to the cell type used [11–14, 33, 34]. In this study with U937 cells, we demonstrated that CHX can induce apoptosis via caspase-dependent pathways and in a dose- but not time-dependent manner. Unprompted and self-imposed inhibition is another important characteristic of this process which resembles classical acquired drug resistance except for its speediness and suddenness, and therefore we have termed it 'sudden drug resistance' (SDR).

Classically, investigations of drug resistance have focused on the single cell by selecting drug-resistant cells following exposure to cytotoxic agents. By and large, these studies have revealed mechanisms that reduce intracellular drug concentration (P-gp, MRP and LRP), detoxify the drug itself (glutathione S-transferase) or alter the drug target (alterations in topoisomerase II) [35]. However, overall protein synthesis inhibition by CHX, as exemplified by unchanged expression of MDR1, c-Fos, c-Jun, Bcl-2, Bcl-x_L and Bax, suggests that SDR may not be related to such mechanisms or to altered protein expression which commonly occurs in the process where CHX inhibits apoptosis induced by other stimuli [11–14]. Instead, our findings have correlated SDR with homotypic aggregation. Removal of bound Mg^{2+} by EDTA, not by EGTA, completely blocks cell-cell adhesion and simultaneously confers an increased sensitivity to CHX-induced U937 apoptosis. Monoclonal antibody directed to CD18 (a common subunit of β_2-integrins) even circumvents SDR. (All these promotions, in combination with sustained full inhibition of protein synthesis by CHX up to 24 h [28], also indirectly demonstrated that CHX might not be reduced and remains effective throughout treatment.) In contrast, the counterpart ligands of β_2-integrins such as sICAM-1 and FB were shown to greatly facilitate SDR by counteracting CHX-induced apoptosis, and the inhibition by RGDS tetrapeptide may be due to its interacting with certain integrins, which subsequently results in increased affinity of integrins [36] and/or activates integrin-linked kinase (ILK) [37]. Together, these observations allow us to suggest that β_2-integrin engagement is a key mediator of SDR, although it may be non-exclusive. With regard to the members of the β_2-integrin family actually functioning to mediate SDR, we did not determine these in detail, since the fact that sICAM-1 and FB, which bind to different members [31], both inhibit apoptosis suggests that LFA-1, Mac-1 and p150,95 expressed in U937 cells might cooperate with each other. The reciprocal effects of serum-free stress and FN also indicate the possibility of cooperative

signalling in the regulation of SDR. In fact, CAM-DR has previously been proposed to explain how some cells survive initial drug exposure and eventually express classical MDR [21]. Our results not only confirm this possibility but also extend this correlation from an integrin-ECM interaction to cell-cell adhesion. More importantly, SDR is an intuitionistic model to display the dynamic process of CAM-DR.

Commonly, cell-cell contacts can be induced by increasing the constitutive expression of β_2-integrin receptor proteins or by regulation of β_2-integrin avidity. Here, we detected no alteration of CD18 surface expression, and therefore surmised that the increase of β_2-integrin avidity might account for homotypic aggregation promoted by CHX. It is well established that the change in avidity is generally accompanied by cytoskeletal reorganization [38, 39] that causes the clustering of β_2-integrin molecules such as LFA-1 and is probably regulated by a Ca^{2+}-dependent protease, calpain [38]. Integrins interact with the actin cytoskeleton via association of their cytoplasmic tails with actin-binding proteins and are constrained in resting cells. Calpain releases them from this constraint to allow integrin clustering upon activation. Hypothetically, the formation of FADD- and caspase-8-containing death-effector filaments that has been shown not to be sufficient for the propagation of a death signal in response to CHX [34] might instead contribute to homotypic aggregation promoted by CHX. However, whether this kind of cytoskeletal change is sufficient to cause β_2-integrin activation and induce homotypic aggregation is still unclear.

After β_2-integrin activation, a phosphorylation cascade would be triggered at the same time. Although little is known about their actual hierarchies, a large number of proximal participants have been identified to be phosphorylated and to function in cell-cell adhesion-mediated signal pathways. Interestingly, all the phosphorylation cases commonly converge on three central roles including PI-3K, PKC and Ras, and then regulate their downstream signalling events [40, 41]. Here, we have simply supported a potential role for PI-3K in the regulation of SDR. Furthermore, active PI-3K can influence cellular apoptotic commitment mainly by activation of Akt, which indirectly stimulates the transcription of pro-survival factors, or directly phosphorylates and inactivates pro-caspase-9 and BAD, a pro-apoptotic member of the Bcl-2 family, to block mitochondrial apoptotic pathways [42]. However, overall protein synthesis inhibition by CHX and no effect on SDR of ASA, which specifically inhibits the activity of IκB kinase-β and hence inhibits NF-κB activation [43], suggest that BAD and/or caspase-9 may really regulate a distal step of SDR. Nevertheless, additional experiments are needed to clarify these mechanisms.

Various integrins are commonly restricted to certain cell types with their own binding specificity and signalling properties [22]. Cooperating with their expression levels, they may well provide another interesting explanation for the selection of cell types with apoptotic sensitivity to a CHX insult. As a good example, HL-60 cells, which were suggested to express LFA-1 but not other β_2-integrin members [44], have been shown to be more susceptible to CHX-induced apoptosis [7, 45]. In some cases, SDR occurrence even masks the ability of CHX to induce apoptosis. At one extreme are Molt-4 and 6T-CEM cells, which undergo apoptosis in response to CHX (10 µg/ml) after pretreatment with EDTA, but make no response under natural or even serum-free incubation (data not shown), probably because of their stronger and more rapid adhesion potential and resistance to apoptosis. These results also raise a caveat with respect to drug screening. It seems to be ill-considered to evaluate whether a chemical is a good candidate for a drug only on the basis of its direct effect, which in some cases can be masked by its other unmonitored properties such as the induction of SDR, rather than by consideration of its overall effect.

In other words, SDR might take place more extensively, especially in leukocytes, but not in all cell types. CHX has been found to cause focal adhesion disassembly in primary aortic endothelial cells [46]. We do not deny the importance of individual diversity of cell types in determining apoptotic sensitivity to the CHX insult. In some cases, both might be concordant. For example, in 6T-CEM cells pretreated with EDTA, CHX-induced apoptosis still occurs slowly and weakly (data not shown). Furthermore, SDR might also extend to front-line chemotherapeutic drugs such as melphalan and doxorubicin. Their cytotoxic effects have been demonstrated to be strongly reduced by FN-mediated adhesion [21]. There may be more such chemicals, so considerations as to which chemicals to use should take into account this inhibition in order to improve clinical outcome.

Obviously, the discovery of SDR per se has profound implications for therapeutic applications. For example, the combination of CHX and inhibitors of cell adhesion may be another feasible approach for leukemia chemotherapy. Recently, the design of small-molecule antagonists of certain integrins such as LFA-1 [47] and VLA-4 [48] provided a material foundation for this approach. On the other hand, other properties of CHX probably independent of cell adhesion are also applicable to reduce the side effects of cancer therapy. Ideally, chemotherapeutic drugs should specifically target only neoplastic cells and should decrease tumour burden by inducing cytotoxic and/or cytostatic effects with minimal ‘collateral damage’ to normal cells. In reality, the effectiveness of chemotherapy has suffered from a range of confounding factors including systemic toxicity due to a lack of specificity, rapid drug metabolism and both intrinsic and acquired drug re-

sistance. Interestingly, the inhibition by CHX of apoptosis induced by other stimuli appears to be dependent on the cell type used. For example, in U937 cells, CHX only partially inhibits VP-16-induced apoptosis, but in Molt-4 and 6T-CEM cells it can do it almost completely (unpublished data). Similar results were also observed with dexamethasone and dibutyryl-cAMP in B and T lymphocytes [33]. These results suggest that relatively low side effects might be possible by combining of CHX and chemotherapeutic agents.

In summary, in this study we have defined a novel form of chemoresistance, sudden drug resistance (SDR), in CHX-induced apoptosis of U937 cells. Unlike classical MDR, SDR occurs rapidly and is mediated, at least in part, by β_2-integrin engagement. As discussed above, SDR would be a new supplement to the classical basis of chemoresistance, and offer another opportunity for improved leukemia therapy.

Acknowledgements. This work was supported by the National Natural Science Fund of China (30271593) and the Basic Research Fund of Tsinghua University (JC2002046).

1 Jacobson M. D., Weil M. and Raff M. C. (1997) Programmed cell death in animal development. Cell **88:** 347–354
2 Hengartner M. O. (2000) The biochemistry of apoptosis. Nature **407:** 770–776
3 Thompson C. (1995) Apoptosis in the pathogenesis and treatment of disease. Science **267:** 1456–1462
4 Hanahan D. and Weinberg R. A. (2000) The hallmarks of cancer. Cell **100:** 57–70
5 Kerr J. F., Wyllie A. H. and Currie A. R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implication in tissue kinetics. Br. J. Cancer **26:** 239–257
6 Lowe S. W. and Lin A. W. (2000) Apoptosis in cancer. Carcinogenesis **21:** 485–495
7 Martin S. J., Lennon S. V., Bonham A. M. and Cotter T. G. (1990) Induction of apoptosis (programmed cell death) in human leukemic HL60 cells by inhibition of RNA or protein synthesis. J. Immunol. **145:** 1859–1867
8 Bicknell G. R., Snowden R. T. and Cohen G. M. (1994) Formation of high molecular mass DNA fragments is a marker of apoptosis in the human leukaemic cell line, U937. J. Cell Sci. **107:** 2483–2489
9 Kartner N., Riordan J. R. and Ling V. (1983) Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. Science **221:** 1285–1288
10 Gottesman M. M. and Pastan I. (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu. Rev. Biochem. **12:** 243–253
11 Martin D. P., Schmidt R. E., DiStefano P. S., Lowry O. H., Carter J. G.. and Johnson E. M. Jr (1988) Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. J. Cell Biol. **106:** 829–844
12 Furukawa K., Estus S., Fu W., Mark R. J. and Mattson M. P. (1997) Neuroprotective action of cycloheximide involves induction of Bcl-2 and antioxidant pathways. J. Cell Biol. **136:** 1137–1149
13 Mattson M. P. and Furukawa K. (1997) Anti-apoptotic actions of cycloheximide: blockage of programmed cell death or induction of programmed cell life? Apoptosis **2:** 257–264
14 Taylor M. H., Buckwalter M. R., Stepheson A. C., Hart J. L., Taylor B. J. and O'Neill K. L. (2002) Radiation-induced apoptosis in MOLT-4 cells requires de novo protein synthesis i ndependent of de novo RNA synthesis. FEBS Lett. **514:** 199–203
15 Ruoslahti E. and Reed J. C. (1994) Anchorage dependence, integrins, and apoptosis. Cell **77:** 477–478
16 Meredith J. E., Fazeli B. and Schwartz M. A. (1993) The extracellular matrix as a cell survival factor. Mol. Biol. Cell **4:** 953–961
17 Frisch S. M. and Francis H. (1994) Disruption of epithelial cell-matrix interactions induces apoptosis. J. Cell Biol. **124:** 619–626
18 Aoudjit F. and Vuori K. (2001) Matrix attachment regulates Fas-induced apoptosis in endothelial cells: a role for c-Flip and implications for anoikis. J. Cell Biol. **152:** 633–643
19 Stupack D. G., Puente X. S., Boutsaboualoy S., Storgard C. M. and Cheresh D. A. (2001) Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. J. Cell Biol. **155:** 459–470
20 Frisch S. M. and Screaton R. A. (2001) Anoikis mechanisms. Curr. Opin. Cell Biol. **13:** 555–562
21 Damiano J. S., Cress A. E., Hazlehurst L. A., Shtil A. A. and Dalton W. S. (1999) Cell adhesion mediated drug resistance (CAM-DR): role of integrins and resistance to apoptosis in human myeloma cell lines. Blood **93:** 1658–1667
22 Hynes R. O. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. Cell **69:** 11–25
23 Gahmberg C. G., Tolvanen M. and Kotovuori P. (1997) Leukocyte adhesion: structure and function of human leukocyte β_2-integrins and their cellular ligands. Eur. J. Biochem. **245:** 215–232
24 Koopman G., Keehnen R. M. J., Lindhout E., Newman W., Shimizu Y., Seventer G. A. van et al. (1994) Adhesion through the LFA-1 (CD11a/CD18)-ICAM-1 (CD54) and the VLA-4 (CD49d)-VCAM-1 (CD106) pathways prevents apoptosis of germinal center B cells. J. Immunol. **152:** 3760-3767
25 Berton G., Fumagalli L., Laudanna C. and Sorio C. (1994) β2 integrin-dependent protein tyrosine phosphorylation and activation of the *FGR* protein tyrosine kinase in human neutrophils. J. Cell Biol. **126:** 1111–1121
26 Fagerholm S., Hilden T. J. and Gahmberg C. G. (2002) Lck tyrosine kinase is important for activation of the CD11a/CD18-integrins in human T lymphocytes. Eur. J. Immunol. **32:** 1670–1678
27 Erhardt P. and Cooper G. M. (1996) Activation of the CPP32 apoptotic protease by distinct signaling pathways with differential sensitivity to Bcl-x_L. J. Biol. Chem. **271:** 17601–17604
28 Liminga G., Martinsson P., Jonsson B., Nygren P. and Larsson R. (2000) Apoptosis induced by calcein acetoxymethyl ester in the human histiocytic lymphoma cell line U-937 GTB. Biochem. Pharmacol. **60:** 1751–1759
29 Klimecki W. T., Futscher B. W., Grogan T. M. and Dalton W. S. (1994) P-glycoprotein expression and function in circulating blood cells from normal volunteers. Blood **83:** 2451–2458
30 Dransfield I., Cabanas C., Alister C. and Hogg N. (1992) Divalent cation regulation of function of the leukocyte integrin LFA-1. J. Cell Biol. **116:** 219–226
31 Plow E. F., Haas T. A., Zhang L., Loftus J. and Smith J. W. (2000) Ligand binding to integrins. J. Biol. Chem. **275:** 21785–21788
32 Jackson S. P., Schoenwaelder S. M., Yuan Y., Rabinowitz I., Salem H. H. and Mitchell C. A. (1994) Adhesion receptor activation of phosphatidylinositol 3-kinase: von Willebrand factor stimulates the cytoskeletal association and activation of phosphatidylinositol 3-kinase and $pp60^{c\text{-}src}$ in human platelets. J. Biol. Chem. **269:** 27093–27099
33 Lemaire C., Andreau K., Souvannavong V. and Adam A. (1999) Specific dual effect of cycloheximide on B lymphocyte apoptosis: involvement of CPP32/caspase-3. Biochem. Pharmacol. **58:** 85–93

34 Tang D., Lahti J. M., Grenet J. and Kidd V. J. (1999) Cycloheximide-induced T-cell death is mediated by a Fas-associated death domain-dependent mechanism. J. Biol. Chem. **274:** 7245–7252
35 Krishna R. and Mayer L. D. (2000) Multidrug resistance (MDR) in cancer mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. Eur. J. Pharm. Sci. **11:** 265– 283
36 Du X., Plow E. F., Frelinger A. III, O'Toole T., Loftus J. C. and Ginsberg M. H. (1991) Ligands 'activate' integrin $\alpha_{IIb}\beta_3$ (platelet GPIIb-IIIa). Cell **65:** 409–416
37 Ortega-Velazquez R., Diez-Marques M. L., Ruiz-Torres M. P., Gonzalez-Rubio M., Rodriguez-Puyol M. and Rodriguez Puyol D. (2003) Arg-Gly-Asp-Ser (RGDS) peptide stimulates transforming growth factor beta1 transcription and secretion through integrin activation. FASEB J. **17:** 1529–1531
38 Elemer G. S. and Edgington T. S. (1994) Microfilament reorganization is associated with functional activation of $\alpha_M\beta_2$ monocytic cells. J. Biol. Chem. **269:** 3159–3166
39 Stewart M. P., McDowall A. and Hogg N. (1998) LFA-1-mediated adhesion is regulated by cytoskeletal restraint and by a Ca^{2+}-dependent protease, calpain. J. Cell Biol. **140:** 699–707
40 Clark E. A. and Brugge J. S. (1995) Integrins and signal transduction pathways: the road taken. Science **268:** 233–239
41 Giancotti F. G. and Ruoslahti E. (1999) Integrin signaling. Science **285:** 1028–1032
42 Yuan J. Y. and Yankner B. A. (2000) Apoptosis in the nervous system. Nature **407:** 802–809
43 Yin M. J., Yamamtoto Y. and Gaynor R. B. (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of Iκ-B kinase-β. Nature **396:** 77–80
44 Hashizume K., Hatanaka Y., Fukuda I., Sano T., Yamaguchi Y., Tani Y. et al. (2002) N-acetyl-L-cysteine suppresses constitutive expression of CD11a/LFA-1α protein in myeloid lineage. Leuk. Res. **26:** 939–944
45 Lu Q. and Mellgren R. L. (1996) Calpain inhibitors and serine protease inhibitors can produce apoptosis in HL-60 cells. Arch. Biochem. Biophys. **334:** 175–181
46 Kabir, J., Lobo M. and Zachary L. (2002) Staurosporine induces endothelial cell apoptosis via focal adhesion kinase dephosphorylation and focal adhesion disassembly independent of focal adhesion kinase proteolysis. Biochem. J. **367:** 145–155
47 Gadek T. R., Burdick D. J., McDowell R. S., Stanley M. S., Marsters J. C. Jr, Paris K. J. et al. (2002) Generation of an LFA-1 antagonist by the transfer of the ICAM-1 immunoregulatory epitope to a small molecule. Science **295:** 1086–1089
48 Porter J. R., Archibald S. C., Brown J. A., Childs K., Critchley D., Head J. C. et al. (2003) Dehydrophenylalanine derivatives as VLA-4 integrin antagonists. Bioorg. Med. Chem. Lett. **13:** 805–808