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Differential sensitivity of colorectal cancer cell lines to artesunate is associated with expression of beta-catenin and E-cadherin

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ABSTRACT

Artesunate, a remarkable antimalarial agent, also reveals profound cytotoxic activity. In the present investigation, we compared the anticancer effects of artesunate on three colorectal cancer cell lines and analyzed the relationship between drug sensitivity and malignant phenotype of the tumor cells. The findings are as follows: poorly-differentiated was colorectal cancer cell line CLY showing nuclear beta-catenin accumulation and loss of E-cadherin; moderately-differentiated were Lovo cells with cytoplasmic distribution of the two proteins; and well-differentiated were HT-29 cells with membranous localization of them. Also, both *in vitro* and *in vivo*, poorly- or moderately-differentiated CLY and Lovo were more susceptible to artesunate treatment than well-differentiated HT-29. Furthermore, the sensitive response of CLY and Lovo to artesunate was associated with membranous translocation of beta-catenin and increased expression of E-cadherin, which indicated the inhibition of hyperactive Wnt signaling pathway and the reversion of the epithelial to mesenchymal transition, respectively. Due to the vital roles of Wnt pathway and the epithelial to mesenchymal transition in tumor differentiation, we thought artesunate could promote the re-differentiation and apoptosis of colorectal cancer cells by inhibition of hyperactive Wnt pathway and reversion of the epithelial to mesenchymal transition.

1. Introduction

Artemisinin and its derivatives have gained considerable interest during the past years as a new generation of antimalarial drugs with no obvious side effects (Klayman, 1985; Hien and White, 1993; Benakis et al., 1997). Recommended by the World Health Organization, the drugs have been used to treat more than 2 million people, mainly in Africa and Asia (Hien and White, 1993). In addition to their tremendous value for malaria treatment, artemisinin and its derivatives also reveal profound cytotoxic activity against tumor cells (Woerdenbag et al., 1993; Singh and Lai, 2001; Efferth et al., 2001, 2003; Posner et al., 2003). Among the artemisinins clinically used for antimalaria treatment, artesunate manifests the greatest cytotoxicity (Efferth and Oesch, 2004). Furthermore, the cytotoxicity of artesunate is independent of multidrug resistance (Efferth et al., 2001), which suggests that artemisinins may be promising for the treatment of refractory tumors. The further investigation in nude mice bearing human xenograft tumors has demonstrated artesunate treatment can strongly retard the growth of Kaposi's sarcoma and colorectal carcinoma (Dell'Eva et al., 2004; Li et al., 2007b). Encouragingly, in the clinical anticancer treatment, artemisinins therapy has been confirmed to be beneficial in controlling disease progression, relieving symptoms, improving life quality and prolonging survival time (Berger et al., 2005; Singh and Panwar, 2006).

Despite its promising future in cancer therapy, anticancer mechanisms of artemisinins are still unresolved. Endoperoxide bridge, the active moiety of artemisinins, is the first clue to explore their mechanisms of action. The cleavage of endoperoxide bridge results in the generation of reactive oxygen species and/or artemisinin carbon-centered free radicals (Meshnick et al., 1993; Posner et al., 1994, 1995). Some investigators have confirmed that the oxidative stress contributes to the anticancer activity of artemisinin drugs (Woerdenbag et al., 1993; Efferth and Oesch, 2004). Furthermore, the genes associated with the oxidative stress response of tumor cells have been identified by microarray assays, including DNA damage and repair genes, apoptosis regulating genes, proliferation-associated genes, oncogenes, tumor suppressor genes, drug resistance genes and angiogenesis-related genes (Efferth et al., 2002; Anfosso et al., 2006). Further investigations have revealed the expression levels of these genes can significantly affect the sensitivity of tumor cells to artemisinins (Efferth et al., 2003; Anfosso et al., 2006). Therefore, we hypothesized some genetic pathways might be involved in the regulation of the series of genes and the activities of these pathways might affect the sensitivity of tumor cells to artemisinins.

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We have previously demonstrated different colorectal cancer cell lines exhibited heterogeneous activities of Wnt signaling pathway (Li et al., 2007a). The evolutionarily conserved Wnt pathway has pivotal roles during the development of many organ systems, and its dysregulation is a key factor for the initiation of various tumors, including colorectal carcinoma (Behrens and Lustig, 2004; Willert and Jones, 2006). One hallmark of Wnt pathway activation is the nuclear accumulation of its main effector beta-catenin (Behrens and Lustig, 2004; Willert and Jones, 2006). Normally, beta-catenin serves as a component of the cytoskeleton, interacting with E-cadherin at the plasma membrane and participating in cell–cell adhesion (Behrens and Lustig, 2004; Willert and Jones, 2006). In normal cells, free cytoplasmic beta-catenin is rapidly phosphorylated, ubiquitinated and degraded (Behrens and Lustig, 2004; Willert and Jones, 2006). In the development of cancer, the mutations of Wnt components decrease the normal ubiquitination and degradation of beta-catenin protein (Behrens and Lustig, 2004; Willert and Jones, 2006). The protein subsequently accumulates in the cytoplasm and translocates to the nucleus, where it binds with transcription factors TCF/LEF to activate transcription of various target genes (Behrens and Lustig, 2004; Willert and Jones, 2006). In addition to activating Wnt pathway, translocation of beta-catenin from adherent junctions to the nucleus can directly induce a loss of E-cadherin and, subsequently, the epithelial to mesenchymal transition (Brabletz et al., 2005a,b). E-cadherin is an intercellular adhesion molecule of epithelial cells, known to play an important role in maintaining the morphological integrity of normal epitheliums (Nelson and Nusse, 2004). The loss of membranous E-cadherin causes the breakdown of epithelial-cell homeostasis and acquisition of an aggressive phenotype, which is referred to as the epithelial to mesenchymal transition and considered to be a crucial event in malignancy (Brabletz et al., 2005a,b). In fact, the intracellular localization and expression of beta-catenin and E-cadherin are very heterogeneous in tumor cells and reflect the heterogeneity of tumor cell differentiation (Brabletz et al., 2005a,b). Therefore, we were interested to determine whether the status of beta-catenin and E-cadherin might affect the sensitivity of tumor cells to artesunate by investigating the effects of artesunate on colorectal cancer cell lines with different malignancy or differentiation.

2. Materials and methods

2.1. Cells and cell culture

Colorectal cancer cell line CLY was established from liver metastasis of a 64-year-old Chinese man with colon adenocarcinoma (Li et al., 2007a). The cell line was characterized with hyperactive Wnt pathway and spontaneous liver metastasis (Li et al., 2007a). Other two colorectal cancer cell lines Lovo and HT-29 were obtained from the American Type Culture Collection (Rockville, MD, USA). Lovo was established from the metastatic nodule resected from a Caucasian man with colon adenocarcinoma and HT-29 from the primary tumor of a Caucasian woman with colon adenocarcinoma. These cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Chemicals and reagents

Artesunate was purchased from the Gulin Pharmaceutical Co. (Guangxi, China). It was prepared as stock solution in dimethyl sulfoxide (DMSO) and diluted with culture medium or sodium chloride injection for *in vitro* study or *in vivo* study, respectively. Control solution contained DMSO at an equivalent (v/v) dilution to that used for the highest concentration of artesunate. RPMI 1640 medium, fetal bovine serum, penicillin, streptomycin and trypsin/EDTA were purchased from Invitrogen (Carlsbad, CA, USA). DMSO, propidium iodine, RNase A, Hoechst 33342, 3-(4,5-dimethylthizol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Mouse anti-human beta-catenin monoclonal antibody, mouse anti-human E-cadherin monoclonal antibody and fluoroscine isothiocyanate-conjugated secondary antibody were obtained from Zymed (San Francisco, CA, USA). Mouse anti-human beta-actin polyclonal antibody, mouse anti-human beta-tubulin polyclonal antibody, the horseradish peroxidase-conjugated secondary antibody and the enhanced chemiluminescence reagent were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyvinyldenefluoride membranes were purchased from Amersham Biosciences (Arlington Heights, IL, USA). Strept avidin biotin complex kit was obtained from Wuhan Boster Bioengineering Ltd. (Wuhan, China).

2.3. Animals

Six-week-old female athymic nude mice (Balb/c nu/nu) were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and housed in specific-pathogen-free conditions in conformity with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

2.4. Growth inhibition assay

Three colorectal cancer cell lines were seeded in 96-well plates at a density of 3000 cells/well. After 24 h culture in the normal growth medium, the cells were exposed to graded concentrations of artesunate for 24, 48 or 72 h respectively. After each time point, the number of viable cells was measured by thiazolyl blue (MTT) assay. Briefly, after incubation with 5 mg/ml MTT solution for 4 h, the cells were dissolved in DMSO and the absorbance at 570 nm was determined by using an ELISA reader (Bio-Tek instrument, Inc., USA). Data were expressed as percentage of viable cell number at a certain time-point compared to viable cell number at time when artesunate was added (time zero in Fig. 1A). IC_{50} value (50% inhibitory concentration) was determined from plots of percent control cell survival vs. log artesunate concentrations (Microcal Origin 5.0 software).

2.5. Flow cytometry analysis

After incubated with different concentrations of artesunate for 72 h, the cells were collected by trypsinization, washed with phosphate buffered saline (PBS) and fixed in 70% ethanol at −20 °C overnight. And then the cells were centrifuged, washed with PBS and treated with 50 μg/ml RNAase at 37 °C for 30 min. After incubation, they were stained with 50 μg/ml propidium iodine. All the samples were analyzed using a FACS Calibur flow cytometer (Becton Dickinson, San Joes, CA, USA) with an excitation wavelength of 488 nm. And the CellQuest software was used to quantify the proportion of the cells within different cell cycle phases and the fraction of the cells exhibiting a DNA content lower than G_1 (apoptotic cells).

2.6. Western blot analysis

After incubated with different concentrations of artesunate for 72 h, the three colorectal cancer cell lines were harvested in ice-cold PBS respectively. Total proteins were extracted and separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and transferred onto polyvinyldenefluoride membrane, as described previously (Gardner et al., 2004). Membranes were blocked with 5% (w/v) dried skimmed milk powder in Tris-buffered saline (blocking solution) for 2 h at room temperature. Then the membranes were probed with primary antibodies against beta-catenin (1/2000 dilution) or E-cadherin (1/2000 dilution) in blocking solution overnight at 4 °C. After a PBS wash, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1/5000 dilution) in blocking solution for 1 h

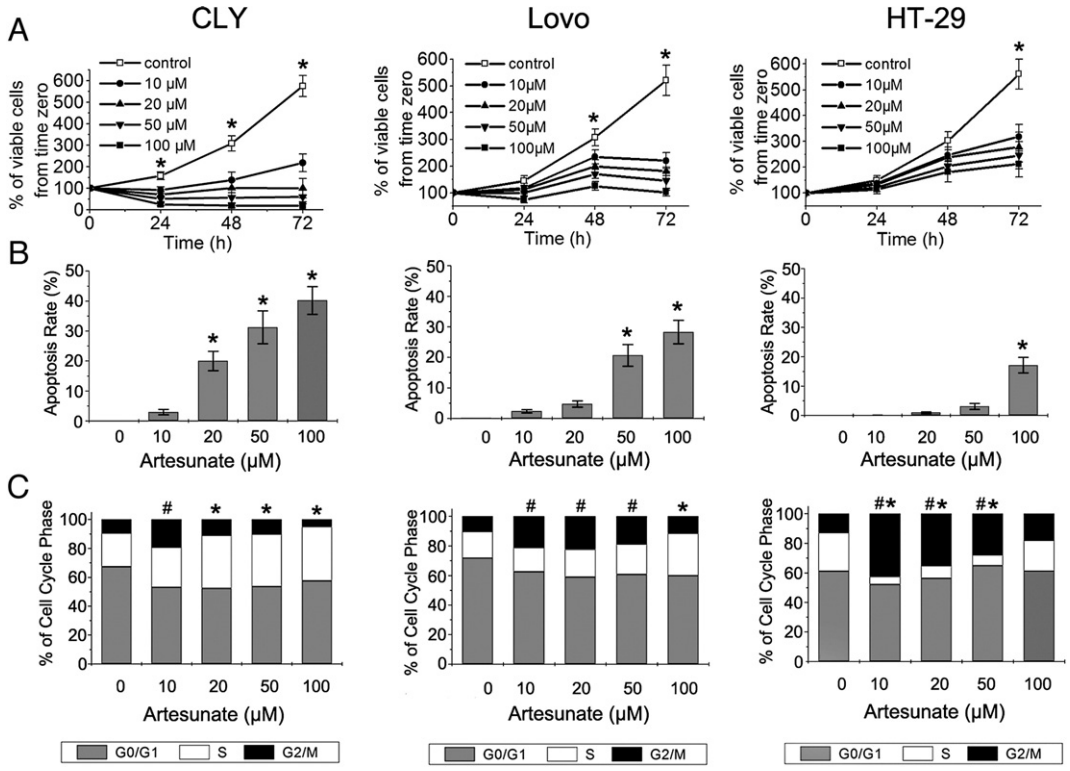

Fig. 1. Effects of artesunate on proliferation, apoptosis and cell cycle parameters of three colorectal cancer cell lines. (A) Artesunate of different concentrations were added to cell cultures at time zero and the number of viable cells was measured by MTT assay at different times later. Data were expressed as viable cell number as percentage from that at time zero. (*) indicates a significant difference from control (Student's *t*-test, $P<0.05$). (B) Following 72 h incubation with different concentrations of artesunate, percent apoptosis of three cell lines was determined by flow cytometry analysis after staining with propidium iodide. The data are shown in a bar graph. (*) indicates a significant difference from control (Student's *t*-test, $P<0.05$). (C) The three colorectal cancer cell lines were treated with different concentrations of artesunate for 72 h. Cell cycle distribution of these samples was measured by flow cytometry and the percent of each cell cycle phase is shown in a bar graph from with the G_0/G_1(gray columns), S(white columns) and G_2/M(black columns) phases for each cell lines. (*) and (#) indicates the difference in the S phase ratio and G_2/M phase ratio compared with control cells, respectively (Student's *t*-test, $P<0.05$). All data were representative of three independent experiments.

at room temperature. Immunoreactive protein was detected using the enhanced chemiluminescence.

2.7. Indirect immunofluorescence analysis

Three colorectal cancer cell lines were grown on glass coverslips in 6-well plates. After artesunate treatment for 72 h, the cells were washed with PBS, fixed with 4% formaldehyde, permeabilized in 0.3% Triton X-100, and blocked in 10% goat serum/PBS. The cells were then stained with anti-beta-catenin or anti-E-cadherin antibody. After a PBS wash, the cells were incubated with fluoroscine isothiocyanate-conjugated secondary antibody. The cells bound with anti-beta-catenin were also counterstained with Hoechst 33342. Images were collected on a Bio-Rad Radiance 2100™ confocal system in conjunction with a Nikon TE300 microscope.

2.8. Anti-tumor effect of artesunate in vivo

Colorectal cancer cells (2×10^6) were injected subcutaneously into the right abdominal flanks of the nude mice. Tumor growth was measured with a slide caliper and the volumes were estimated according to the following formula: Tumor volume (mm^3)$=L\times W^2/2$, where L is length and W is width. When the tumor volume reached about 100 mm^3, the mice were sorted into 2 groups ($n=7$) with almost mean equal tumor sizes and administration was started. Artesunate-treated mice were administered by intravenous injection at a dose of 300 mg/kg twice a week. Control mice were dosed with vehicle solution at the same time. The experiment was stopped when the tumor volumes of the control mice reached about 1000 mm^3. At the end of the treatment, the mice were sacrificed for autopsy and the tumors were recovered and weighted. The tumor growth inhibitory rate was calculated as follow: Inhibitory Rate (%)=[1−(mean tumor weight of treated group)/(mean tumor weight of control group)]× 100. The significance of differences was determined by the Student's *t*-test. In order to confirm the effects of artesunate on beta-catenin localization and E-cadherin expression on tissue levels, the immunohistochemical staining of tumor tissues was carried out according to Wong et al. (2004). Strept avidin biotin complex kit was used as a secondary reagent. Stainings were developed using 3,3′-diaminobenzidine (DAB, brown precipitate). Slides were counterstained with hematoxylin.

3. Results

3.1. Effects of artesunate on cell proliferation, apoptosis and cell cycle parameters

Artesunate inhibited the growth of the three colorectal cancer cell lines in concentration- and time-dependent manner by the MTT assay (Fig. 1A). However, there existed marked differences among the effects of artesunate on individual cell lines. In the absence of artesunate, there was a 500–600% increase in viable cell number over 72 h. At the concentrations above 20 μM, artesunate caused complete inhibition of proliferation of CLY cells. However, the inhibitory effect of artesunate was not as marked on Lovo cells which were completely inhibited at the concentration of 100 μM. As to HT-29 cells, artesunate had a partial inhibitory effects but there was still significant cell proliferation, even in the presence of the highest concentration of artesunate. The IC_{50} values of CLY, Lovo and HT-29 at 72 h were 20.34±2.20 μM, 30.55± 0.73 μM and 82.34±3.74 μM, respectively.

Flow cytometry analysis also demonstrated the apoptosis rates of the three colorectal cancer cell lines increased with artesunate concentration increasing (Fig. 1B). Similarly, artesunate had the strongest apoptosis-promoting effect on CLY cells and the weakest effect on HT-29 cells. At the same time, artesunate induced similar cell cycle changes in all three cell lines (Fig. 1C). Lower concentrations of artesunate mainly caused G_2/M arrest, which was more significant in HT-29 cells. With concentration increasing, artesunate decreased the proportion of the cells in G_2/M phase and increased cells in S phase. Of the three colorectal cancer cell lines, CLY showed the most prominent effects on S phase arrest and apoptosis at higher concentrations of artesunate.

3.2. Anticancer effects of artesunate in vivo

Colorectal cancer cells were injected subcutaneously into the flanks of nude mice. When the tumor volumes reached about 100 mm^3, the mice were sorted into 2 groups (n=7) and administration was started. Tumor volumes of the two groups, as one index to evaluate the treatment, were measured and compared every 2 day (for CLY and HT-29) or every day (for Lovo). Statistical significant differences (P<0.01), if compared with the control tumor volumes, were reached from day 9 on for CLY tumors and day 6 on for Lovo tumors (Fig. 2A). As to HT-29 tumors, artesunate also inhibited the increasing of tumor volumes, although the inhibitory effects did not attain statistical significance (Fig. 2A). At the end of the experiment, the tumors were resected and weighted. For CLY, the average tumor weight of artesunate-treated mice was 457±87 mg, which was much less than that of control mice (924±177 mg) (Fig. 2B). The tumor growth inhibitory rate was 50.5% (P=0.000042). Similarly, artesunate significantly inhibited the growth of Lovo xenograft tumors. The tumor weight of control group and treatment group were 1320± 300 mg and 631±150 mg, respectively (Fig. 2B). The tumor growth inhibitory rate amounted to 52.2% (P=0.00052). However, HT-29 xenograft tumors were much less sensitive to artesunate treatment. The tumor weight of control group was 675±146 mg, which didn't exhibit statistically significant difference from artesunate-treated group (544±98 mg, P=0.072) (Fig. 2B).

3.3. Effect of artesunate on protein levels and subcellular localization of beta-catenin

Previously, we have demonstrated that different colorectal cancer cells have different beta-catenin protein expression and localization (Li et al., 2007a). Therefore, we were interested to determine whether the discrepant sensitivity of different colorectal cancer cells to artesunate described above were associated with changes in the expression and localization of beta-catenin. According to western blot analysis, there were no significant changes in beta-catenin protein levels at concentrations below 100 μM in all three colorectal cancer cells (Fig. 3A). However, 200 μM artesunate caused 48% and 55% reductions in beta-catenin protein levels in CLY and Lovo cells, respectively (Fig. 3A). As to HT-29 cells, no significant changes in beta-catenin protein levels were observed, even at the highest concentration of artesunate (Fig. 3A).

Indirect immunofluorescence studies of beta-catenin localization in CLY cells revealed that treatment with 50 μM artesunate for 72 h was associated with a reduction in nuclear beta-catenin and a concurrent increase in beta-catenin protein expression at cell–cell contacts compared with cells treated with DMSO alone (Fig. 3B). Lovo cells, under the same conditions, exhibited a reduction in cytoplasmic beta-catenin immunofluorescence signal and a corresponding increase in membranous beta-catenin localization (Fig. 3B). However, beta-catenin demonstrated a strongly restricted membranous distribution in HT-29 cells and no significant changes in subcellular localization following artesunate treatment (Fig. 3B).

The results of immunohistochemistry of xenograft tumors substantiated the effects of artesunate on beta-catenin subcellular translocation. Following artesunate treatment, the nuclear beta-catenin of CLY tumor tissues and cytoplasmic beta-catenin of Lovo tumor tissues all translocated to membrane (Fig. 3C). However, beta-catenin demonstrated membranous staining in HT-29 tumor issues and artesunate treatment did not alter its subcellular distribution (Fig. 3C).

3.4. Effect of artesunate on protein levels and subcellular localization of E-cadherin

Our previous study demonstrated that E-cadherin had a highly restricted membranous distribution in HT-29 cells, a dispersed cytoplasmic and membranous distribution in Lovo cells and no significant expression in CLY cells (Li et al., 2007a). Therefore, we were interested to determine whether the expression and localization of E-cadherin significantly influenced the sensitivity of the different colorectal cancer cells to artesunate and whether E-cadherin had corresponding changes following beta-catenin alterations after artesunate treatment. According to western blot analysis, in the absence of artesunate, there were a weak expression in CLY cells and a strong expression in Lovo and HT-29 cells (Fig. 4A). CLY cells just had a 25% or

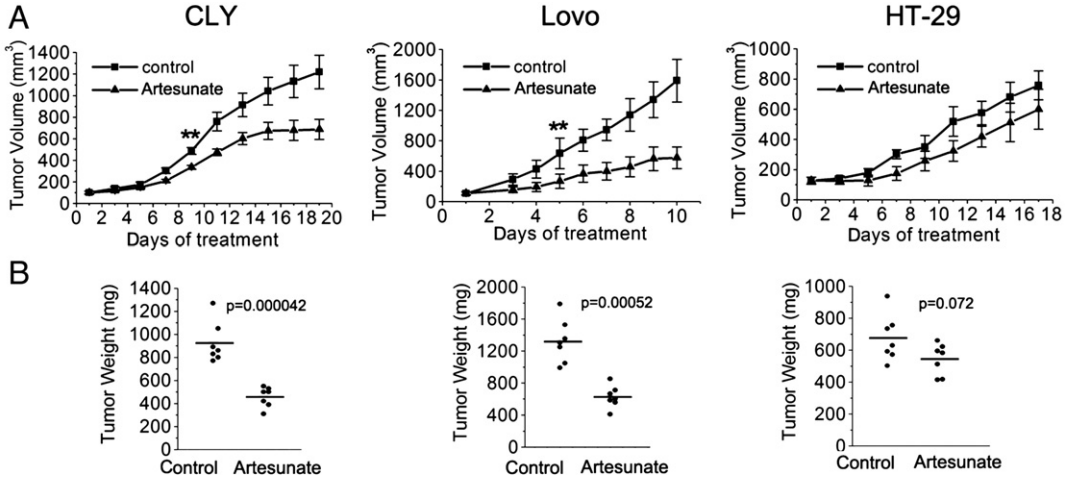

Fig. 2. Anti-cancer effects of artesunate *in vivo*. Colorectal cancer cells were injected into the flanks of nude mice. When the tumor volume reached about 100 mm^3, the mice were sorted into 2 groups (n=7) and administration started. (A) Growth difference of tumor volumes was significant from day 9 on for CLY tumors and day 6 on for Lovo tumors. (**) indicates a significant difference from control (Student's t-test, P<0.01). (B) At the end of the experiment, tumors were resected and weighted. (●) indicates the weight value of each tumor; (—) indicates average value of tumor weights.

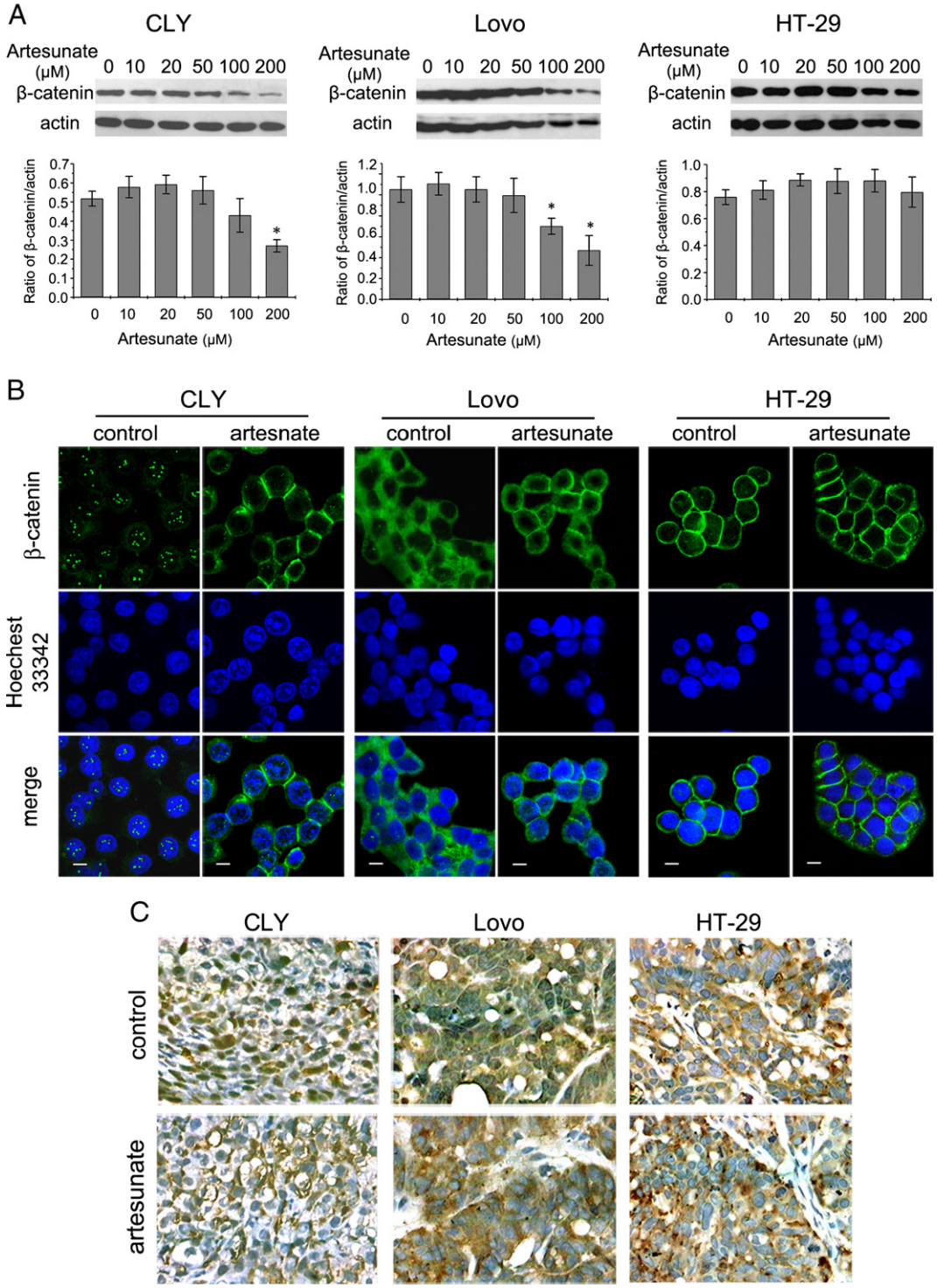

Fig. 3. Effect of artesunate on protein levels and subcellular localization of beta-catenin. (A) Western blot analysis of beta-catenin (92 kDa) and beta-actin (42 kDa) protein expression in three cell lines was carried out after 72 h incubation with differing concentrations of artesunate. Expression of beta-actin was used as an internal control. A representative blot for each cell lines is accompanied by a quantitative analysis of triplicate beta-catenin blots. Densitometric data are expressed as the mean (+S.E.M.) percentage of the control beta-catenin/beta-actin ratio. (*) indicates the difference in the beta-catenin/beta-actin ratio compared with control cells (Student's t test, $P<0.05$). (B) Indirect immunofluorescence analysis of beta-catenin in three cell lines was performed following 72 h treatment with 50 μM artesunate. beta-catenin labeling green and nucleus stained by Hoechst 33342 labeling blue. Bar = 10 μm. (C) Immunohistochemistry analysis of beta-catenin in three colorectal cancer xenograft tumors. (original magnification, ×220). All data were representative of three independent experiments.

33% E-cadherin protein level of Lovo or HT-29 cells. After artesunate treatment for 72 h, CLY cells showed a concentration-dependent increase in E-cadherin protein levels, which reached statistical significance at concentration equal to and above 20 μM (Fig. 4A). Similar change in Lovo cells was evident following the same treatment, although the change was just statistically significant at higher concentrations (above 100 μM) (Fig. 4A). However, as to HT-29 cells, there was a 25% to 33% reduction in E-cadherin protein levels after 72 h incubation with 50–200 μM artesunate, which was statistically significant (Fig. 4A).

Immunofluorescence detection of E-cadherin in CLY cells revealed that membranous E-cadherin immunofluorescence signal intensified

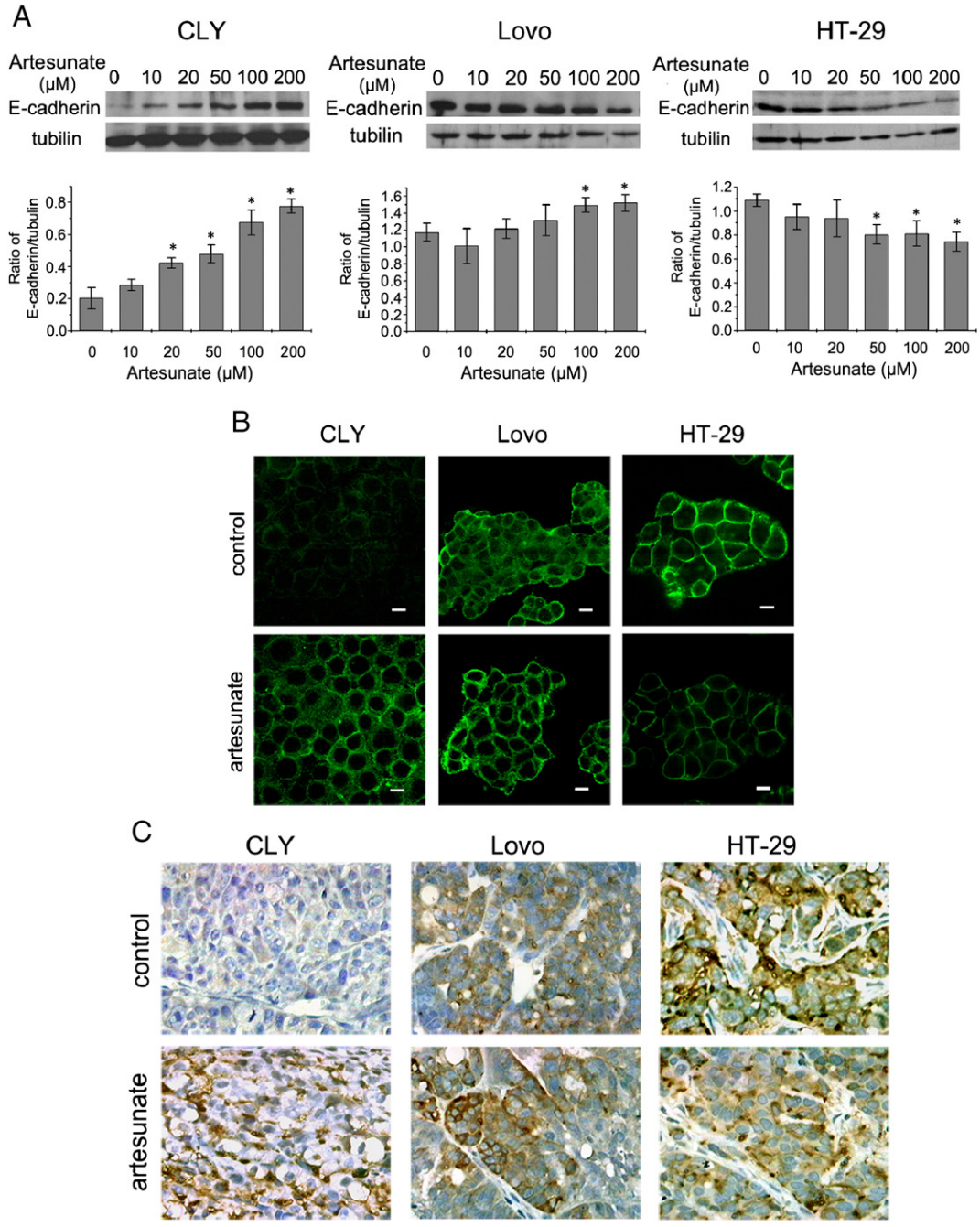

Fig. 4. Effect of artesunate on E-cadherin expression. (A) All the three colorectal cancer cells were treated with different concentrations of artesunate for 72 h. The protein levels of E-cadherin (120 kDa) in these samples were then determined by Western blot analysis. beta-Tubulin (55 kDa) was used as an loading control. A representative blot for each cell lines is accompanied by a quantitative analysis of triplicate E-cadherin blots. The band intensity was measured by densitometric analysis and represented as the mean (+S.E.M.) percentage of the control E-cadherin/beta-tubulin ratio. (*) indicates the difference in the E-cadherin/beta-tubulin ratio compared with control cells (Student's *t*-test, $P<0.05$). (B) Indirect immunofluorescence detection of E-cadherin in three cell lines treated with 50 μM artesunate for 72 h. Bar = 10 μm. (C) Immunohistochemistry analysis of E-cadherin in the three colorectal cancer xenograft tumors. (original magnification, ×220). All data were representative of three independent experiments.

significantly after treatment with 50 μM artesunate for 72 h (Fig. 4B). The same treatment in Lovo cells was associated with decreased cytoplasmic staining and predominantly membranous localization (Fig. 4B). On the contrary, HT-29 cells, under the same conditions, exhibited a decreased membranous immunofluorescence signal (Fig. 4B).

Immunohistochemistry of xenograft tumors confirmed the effects of artesunate on E-cadherin mentioned above. Following artesunate treatment, there was a significantly enhanced membranous staining in both CLY and Lovo tumor tissues (Fig. 4C). However, the same treatment had a different effect on HT-29 xenograft tumors. Artesunate-treated HT-29 tumor tissues demonstrated an obvious reduction in membranous staining (Fig. 4C).

4. Discussion

In the present investigation, we compared the anticancer effects of artesunate on three colorectal cancer cell lines and analyzed the relationship between drug sensitivity and malignant phenotype of tumor cells. Colorectal cancer cell line CLY, Lovo and HT-29 were established from metastatic liver, metastatic nodule and primary tumor of patients with colorectal adenocarcinoma, respectively. In

terms of the origin of these cell lines, CLY and Lovo were more malignant than HT-29. In the molecular levels, CLY and Lovo also exhibited more malignant phenotypes than HT-29. Beta-catenin, the main effector of Wnt pathway, demonstrated a definite nuclear localization, a dispersed cytoplasmic accumulation and a highly restricted membranous distribution in CLY, Lovo and HT-29, which signified Wnt pathway was hyperactive, moderate active and inactive in the three cell lines, respectively. In addition to the activation of Wnt pathway, beta-catenin nuclear accumulation also induces the loss of membranous E-cadherin, which is an important hallmark of the epithelial to mesenchymal transition (Brabletz et al., 2005a,b) and was observed in CLY cells. However, HT-29 cells and Lovo cells showed an obvious expression of E-cadherin, which indicated the invasive malignancy of Lovo and HT-29 was weaker than that of CLY. Taken together, of the three colorectal cancer cell lines, CLY had the most malignant phenotypes and HT-29 had the least one. After artesunate treatment, both *in vitro* and *in vivo*, the three colorectal cancer cell lines all demonstrated growth inhibition. However, there were marked differences in the anticancer effects of artesunate on individual cell lines. CLY exhibited the most sensitive response to artesunate treatment and HT-29 showed a relative insensitive response to the same treatment. Therefore, the differential sensitivities of the colorectal cancer cell lines to artesunate may be associated with the differentiation degrees of these cell lines.

Our further investigation indicated the different sensitivity of these colorectal cancer cell lines to artesunate was associated with the inhibitory effects of artesunate on their differentially active Wnt pathway. The Wnt pathway has emerged as a critical regulator of stem cell (Reya and Clevers, 2005). In the crypts of colorectal mucosa, the signaling appears to regulate epithelial renewal through controlling a coordinated series of events involving proliferation, differentiation, and migration up to the luminal surface (Johnston et al., 2007). At the bottom of the crypt, the stem cells accumulate nuclear beta-catenin and have the unique ability to self-renew (van de Wetering et al., 2002; Reya and Clevers, 2005; Johnston et al., 2007). As the cells proceed up to the mid-crypt region, nuclear beta-catenin gradually translocates to membrane and the activity of Wnt pathway is correspondingly downregulated, which results in cell cycle arrest and differentiation (van de Wetering et al., 2002; Reya and Clevers, 2005; Johnston et al., 2007). Once at the top, the cells undergo apoptosis and/or extrusion into lumen (van de Wetering et al., 2002; Reya and Clevers, 2005; Johnston et al., 2007). The fine balance between cell proliferation and differentiation is controlled by the Wnt pathway and any perturbation of the balance appears to disturb normal intestinal homeostasis leading to tumor development (van de Wetering et al., 2002; Reya and Clevers, 2005; Johnston et al., 2007). In our investigation, CLY cells characterized with hyperactive Wnt pathway were most sensitive to artesunate. After artesunate treatment, the nuclear beta-catenin of CLY cells translocated to membrane, which implicated the tumor cells might cease proliferating and begin to differentiation following artesunate treatment. Due to the re-differentiation, the malignant tumor cells might lose stem cell-like characters, such as self-renew and directional migration, which finally resulted in the losing of limitless proliferating ability and metastatic potential. Some animal experiments and clinical case reports have demonstrated the inhibitory effects of artesunate on tumor growth and metastasis (Berger et al., 2005; Li et al., 2007b). Therefore, artesunate may promote stem cell-like malignant tumor cells proceeding to differentiation and apoptosis through inhibiting the hyperactive Wnt pathway. However, well-differentiated tumor cells with inactive Wnt pathway, such as HT-29, were not sensitive to artesunate. So it seemed that tumor cells relying on hyperactive Wnt pathway were more sensitive to artesunate. In addition, some investigators have used comparative genomic hybridization technique to identify genomic regions that are associated with drug sensitivity and resistance and found gene *FZD7* (Frizzled homologue 7, a receptor of Wnt pathway) located at 2q33 may affect the action of artesunate (Efferth et al., 2004). Therefore, the relationship between Wnt pathway and anticancer mechanism of artesunate may be much closer and more significant than we understand at present.

In the comparative genomic hybridization experiment mentioned above, the researchers also observed gene *CDH1* (E-cadherin) located at 16q22.1 might contribute to sensitivity or resistance to artesunate (Efferth et al., 2004). In the present investigation, we analyzed the relationship between E-cadherin and anticancer mechanism of artesunate on cell level and tissue level. Tumor cells with low E-cadherin expression, such as CLY, demonstrated a most sensitive response to artesunate and showed a significantly enhanced membranous expression of E-cadherin after artesunate treatment. As the loss of membranous E-cadherin directly induced by beta-catenin activation is an important hallmark of the epithelial to mesenchymal transition (Brabletz et al., 2005a,b), the re-expression of E-cadherin following beta-catenin membranous translocation in artesunate-treated CLY cells and xenograft tumors was suggestive of the mesenchymal to epithelial transition, a reversible process of the epithelial to mesenchymal transition. The epithelial to mesenchymal transition and the mesenchymal to epithelial transition are reversible and dynamic processes, which are signs of tumor de-differentiation and re-differentiation, respectively, and can be regulated by interactions of tumor cells with their microenvironment (Brabletz et al., 2005a,b). Therefore, we hypothesized artesunate might affect tumor microenvironment, which resulted in the reversion of the epithelial to mesenchymal transition and then the re-differentiation and apoptosis of tumor cells. However, well-differentiated HT-29 revealed a decreasing E-cadherin expression after artesunate treatment, which was contrary to the effects on poorly-differentiated CLY and moderately-differentiated Lovo cells. These results suggested the individual microenvironments of variously differentiated tumors might be different and be differently regulated following artesunate treatment. Therefore, it is very necessary to emphasize individualized cancer treatment in further clinical trials.

In addition, artesunate seems to be an inhibitor of differentiation in haematopoietic tumor cells. Some researchers have reported that artesunate could inhibit the production of heme in mouse erythroleukemia cells and therefore block the erythroid differentiation (Kelter et al., 2007). The discrepant effects of artesunate on the differentiation of different kinds of tumors further confirm the significance of individualized cancer treatment and are worthy of more investigations.

As to the relationship between cell proliferative activity and drug sensitivity, some investigators have reported that cell lines with a low percentage of G_0/G_1 cells and a high percentage of S phase cells have a high proliferative activity and are most sensitive to artesunate (Efferth et al., 2003). In our study, the three colorectal cancer cell lines did not show noticeable differences in cell proliferation and cell cycle distribution. Nevertheless, there were significant differences in anticancer effects and cell cycle distribution following artesunate treatment. CLY predominantly demonstrated an accumulation in S phase, while HT-29 mainly showed an arrest in G_2/M phase. The G_2/M arrest serves to allow time for repairing damage, which then results in resumption of proliferation or, if the damage is too severe, senescence to prevent further genetic instability (Dasika et al., 1999; Kastan and Bartek, 2004). Although this mechanism is beneficial to non-neoplastic tissues, in tumor cells it can promote resistance to therapies that rely on eliminating proliferating cells (Crosby et al., 2007). In our study, lower concentrations of artesunate mainly caused G_2/M arrest and the effect was more significant in HT-29 cells, which indicated these cell lines were less sensitive to low concentrations of artesunate and HT-29 was more resistant to artesunate. With increasing concentration of artesunate, S phase arrest was predominant and apoptosis rate increased, which was more prominent in CLY cells. The S phase arrest induced by artesunate was most likely to be the consequence of irreversible damage. It was possible that part of such

cells were damaged beyond their reparative capacity and then progressed to apoptosis. These results suggested the possibility that different cellular targets were being affected, depending on the concentrations of artesunate or, alternatively, that a higher number of similar lesions induced different consequences. At all events, the three colorectal cancer cell lines were more sensitive to higher concentrations of artesunate and CLY was the most sensitive one in these cell lines. These results substantiated the relationship between the differentiation degrees of colorectal cancer cell lines and their different responses to artesunate.

In summary, colorectal cancer cell lines with different malignant phenotypes demonstrated differential sensitivity to artesunate. Poorly-differentiated colorectal cancer cells with nuclear beta-catenin accumulation and no membranous E-cadherin, such as CLY cells, were of great sensitivity to artesunate treatment; moderately-differentiated Lovo cells with cytoplasmic distribution of the two proteins were also sensitive to artesunate; while well-differentiated HT-29 cells showing membranous localization of them were much less sensitive to the same treatment. Furthermore, we substantiated the differentiate sensitivity of colorectal cancer cells to artesunate was associated with the inhibition of hyperactive Wnt pathway and the reversion of the epithelial to mesenchymal transition. We hope these results and further extensive studies will benefit individualized colorectal cancer treatment in clinical research.

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