The effect of insulin on chemotherapeutic drug sensitivity in human esophageal and lung cancer cells

Published in: Natl Med J China, February 10, 2003; Vol 83, No 3, Page 195-197.

Authors: JIAO Shun-Chang, HUANG Jing, SUN Yan, LU Shi-Xin

Affiliations: JIAO Shun-Chang, Department of Oncology, Liberation Army General Hospital; HUANG Jing and SUN Yan, Department of Medicine, Hospital of Oncology, Chinese Academy of Medical Sciences; LU Shi-Xin, Etiology Department, Institute of Cancer Research, Chinese Academy of Medical Sciences; LU Shi-Xin, Institute of Cancer Research, Chinese Academy of Medical Sciences. Beijing, 100853.

[Abstract]

Objective To explore the effect of insulin as a metabolic promoter on chemotherapeutic drug sensitivity in human esophageal and lung cancer cells.

Methods Human esophageal cancer NEC cells and human lung adenocarcinoma GLC cells were cultured and then inoculated into wells. Chemotherapeutic drug etopside, cisplatin, or 5-fluorouracil (5-FU) was added to examine its cytotoxicity. Then, insulin at the final concentration of 5 mU/ml was added 8 hr before etopside (30-40 μg/ml), cisplatin (2.5 μg/ml), or 5-FU (50 μg/ml) was added. MTT method was used and the value was tested by colorimetry to evaluate the number of cancer cells, cell activity, and metabolism status reflecting the cytotoxicity of the anti-tumor agent. Insulin was added into the suspension of cancer cells. Flow cytometry was used to detect the cell-cycle progress.

Results Insulin alone did not inhibit the cell growth and mildly promoted the cell metabolism with the concentration > 5 mU/ml. Insulin (2.0-15.0 mU/ml) enhanced the chemocytotoxicity of etopside (30 μg/ml) on human esophageal and lung cancer cells as indicated by MTT colorimetry. GLC cell cycle assay showed that the S phase block (or arrest) induced by etopside, cisplatin, and 5-FU and the G_2/M block induced by 5-FU were enhanced by insulin with the increased block rates of 80% and 90%, respectively. The increased block rate induced by insulin in NEC cells was lower than in GLC cells.

Conclusion As a reversible metabolic promoter, insulin enhances the cytotoxity of the chemotherapeutic agents. It is possible to increase the growth and metabolism of cancer cells first, in order to enhance the chemosensibility, and then administer chemotherapeutic agents, thus improving their therapeutic effects.

[Key Words] Esophageal neoplasms; Lung neoplasms; Chemotherapeutic sensitivity; Insulin

Modulating tumor cells to restore their activity or increase their sensitivity to chemotherapeutic drugs is a hot research point to increase the efficacy in the comprehensive anticancer therapy. Due to different mechanisms of action, many different methods can be used to increase a chemotherapeutic drugs' sensitivity, such as immunomodulation, gene engineering modulation, and biochemical and physical approaches. However, to date, not many efforts have been shown definitively to increase the chemotherapeutic sensitivity. In an extended period of oncological clinical practice, we observed one phenomenon, i.e., highly differentiated, lowly malignant tumors had low chemotherapeutic sensitivity, while lowly differentiated, highly malignant tumors had high chemotherapeutic sensitivity. Some reasons were that lowly differentiated cells had active proliferation, high metabolic rate, and thus these cells were easily damaged by cytotoxic drugs. On other hand, highly differentiated cells often stayed in the G_0 phase with low metabolic rate and therefore these cells were damaged less by cytotoxic drugs. Based on the mechanisms of chemotherapy, it would be possible to increase a chemotherapeutic drugs' sensitivity by increasing the metabolic rate of tumor cells first, then to treat these cells with chemotherapeutic drugs. Based on the above hypothesis, this study used insulin as a metabolic promoter to induce the activity of chemotherapeutic drugs. The study explored new pathways to increase chemotherapeutic drug sensitivity via cell metabolism, cell cycle [1], and so on.

Materials and Methods

I. Materials

CO_2 incubator, LNA-111DH TABA (Japan); Microplate Reader, EL 340 (U.S.A.); Inverted microscopy (Olympus); Flow cytometer, EPICS ELITE ESP (Coulter). Major reagents, MMT, Pancreatic enzymes (Sigma), cell culture medium DMEM, and bovine serum (GIBcoRnase Boehringe Mannhein); PI, propidium iodide; PBS preparation, 10 mg/ml. Chemotherapeutic drugs, etopside, cisplatin, or 5-fluorouracil (5-FU), prepared with sterile saline solution in desired concentrations before application. Insulin, as a promoter (or inducer, or enhancer), 40 IU/ml, kept in the refrigerator at 4°C, and diluted to the desired concentrations before use. Cancer cells: GLC, human lung adenocarcinoma cells; NEC, human embryo esophageal cancer cells; these cells were prepared by the Etiology Department, Institute of Cancer Research, Chinese Academy of Medical Sciences.

II. Methods

1. Cell culture: Human esophageal cancer cells (NEC) and human lung adenocarcinoma cells (GLC) were obtained as a gift from the Etiology Department, Institute of Cancer Research, Chinese Academy of Medical Sciences. Using the DMEN culture medium containing 10% bovine serum, 100U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO_2 incubator, the cells proliferated adherent to the wall, 3-4 days per generation.

2. MTT colorimetry analytical method was used to determine cell viability (metabolic rate): After the culture cells were routinely digested, they were inoculated into the 96-well culture plate at $5x10^4$/well/200 μl. Four hours before the conclusion of the experiment, 10 μl/well of MTT at 5 mg/ml was added and continued to culture for 4 hr. Discarding the supernatant, 200 μl/well of DMSO was then added. After MTT metabolite Formazan totally dissolved, a colorimeter (U.S.A.) was used at single wavelength of 492 nm to determine A (OD) value of

each well, and repeated with 4-6 wells in each group. There was a linear relationship between the MTT A value and the number of inoculated cancer cells. This value reflected the cells' metabolic activity and reflected the in vitro chemotherapeutic drugs' cytotoxity. Cell inhibition rate (%) = (1 – A value of experimental group / A value of control group) x 100%. Pilot study data showed that when the GLC cancer cells inoculation number was $2x10^3$-$6x10^4$/ml at 200 μl/well, there was a linear relationship with the MTT metabolic A value, and at the same time reflected cell metabolic activity. This also could be used to evaluate the in vitro chemotherapeutic drugs' cytotoxity.

3. Effects of chemotherapeutic drugs and promoter's enhancing activity: A certain number of cells was inoculated into a culture plate and cultured for 24 hr followed by experimental evaluations. After adding chemotherapeutic drugs for 90-120 min and discarding the supernatant, fresh culture solution was exchanged and experimental observation was continued. The solution was not changed after adding the promoter. The concentrations of the chemotherapeutic drugs and the promoter varied based on experimental design. Pilot study data showed that cisplatin and etopside had good concentration-effect linear relationships, but the relationship was not very linear with 5-FU. The concentrations selected for cisplatin 2.5 μg/ml and etopside 30-40 μg/ml were the in vitro concentrations and they are equivalent to human plasma concentrations.

4. Cell cycle and apoptosis were determined by flow cytometry: The culture cells were routinely digested, becoming suspended in solution. Then, $5x10^5$-$1x10^6$ cells were transferred into a centrifuge tube, centrifuged at 800-1000 r/min for 5 min, washed with PBS twice, centrifuged at 800-1000 r/min for 5 min, and the supernatant was discarded. Approximately 0.5 ml PBS stayed in the tube, mixed well with the cells. Next, 70% alcohol was added into the suspended cell solution, mixed quickly, and placed in 4°C for over 8 hr. The solution was then centrifuged at 800-1000 r/min for 5-10 min, the alcohol was discarded, a suitable amount of PBS suspended cells were added, centrifuged at 800-1000 r/min and washed once. Then, 0.4 ml PBS suspended cells were kept and 5 μl (10 mg/ml) of RNA enzyme were added, placed in 37°C for 1 hr then PI (100 μg/ml) was added. Flow cytometry was used to determine cell phase block (or arrest). Cell cycle phase arrest rate (%) = (Cell cycle phase arrest number in experimental group / Cell cycle phase arrest number in control group -1) x 100%. Cell cycle arrest represented the cytotoxic effect.

Results

I. Chemotherapeutic drug enhanced by insulin.

Inoculated cells: GLC $4x10^5$/well; chemotherapeutic drug, etopside, final concentration 30 μg/ml; insulin 1:3 diluted, added 8 hr before etopside addition. Data showed that insulin alone did not inhibit the cell growth; when its concentration was greater than 5 mU/ml, insulin had a slight effect in increasing metabolism, manifested by a negative inhibition rate; when used in combination with etopside, insulin concentration at 0-2 mU/ml had concentration-dependent effect in enhancing the etopside's cytotoxicity. The best enhancing concentration was from approximately 2-15 mU/ml. When >20 mU/ml, the enhancing effect reduced, probably related to the saturation of insulin receptors. This suggested that insulin, which promoted metabolism, enhanced etopside's effect on the cells.

II. Effects of chemotherapeutic drugs and the promoter on cell cycles.

1. Effect of chemotherapeutic drugs on cell cycles: Inoculation of GLC or NEC cells into 24-well plate: $6x10^5$/well/1 ml, streptomycin, at 37°C, 5% CO_2, cells were collected 24 hr after chemotherapeutic drug treatment for cell cycle analysis. In the GLC experiment, when the concentration of etopside increased, cell number in G_2/M phase increased, apoptotic cells increased and cell number in S phase showed a tendency of increase. Etopside's cytotoxicity mainly manifested in the arrest of G_2/M phase and S phase. After cisplatin treatment, when the concentration of cisplatin increased, cell number in G_2/M phase increased, followed by S phase arrest increase. When cisplatin concentration was 2.5 μg/ml, cell number in S phase and G_2/M phase increased when treatment was prolonged. For NEC cells, when cisplatin concentration increased (0-12.5 μg/ml, evaluated 48 hr after treatment) there was a tendency for the cell number to increase in G_2/M phase. When 5-FU concentration was greater than 50 mg/ml, GLC cell number increased in the S phase, responding to the increase in 5-FU concentration. When the 5-FU concentration increased to 250 mg/ml, cell number in S phase increased obviously, suggesting that GLC cells had a low sensitivity to 5-FU. For NEC cells, 5-FU's inhibitory effect was on the arrest in S phase.

2. Effect of insulin on cell cycles: In the GLC cell experiment, insulin at 0-500 mU/ml for 12 hr showed a tendency to increase cells in S phase. When the final insulin concentrations were from 5-50 mU/ml for 0-8 hr, along with increasing through time, cell number in S phase increased gradually. After 8 hr, the effect gradually approached the control level, suggesting that the effect of insulin had a short-term effect and this effect was reversible. In the NEC cell experiment, when insulin concentrations increased (0.05-500 mU/ml, 24 hr), there was a tendency of increase in cell number in S phase. When insulin final concentrations were from 5-50 mU/ml, during 0-24 hr, along with increasing through time, cell number in S phase increased gradually. After 24 hr, the cell number gradually decreased, even less than the control level. This observation was similar with what observed in the GLC cell experiment.

3. Enhancing effect of insulin on chemotherapeutic drugs' cytotoxicity: Inoculation of GLC or NEC cells into a 24-well plate, $5x10^5$/well/1 ml (repeated 4 wells for each group). For the control, only chemotherapeutic drugs were added. For experimental groups, 8 hr after insulin was added, chemotherapeutic drugs were added. The final concentration for insulin was 5 mU/ml, for cisplatin was 2.5 μg/ml [1 PPC (peak plasma concentration)] = 2.5 μg/ml, for 5-FU was 50 μg/ml [1 PPC = 50 μg/ml], and for etopside was 30 μg/ml [1 PPC = 30-40 μg/ml]. Experimental details have been described in the Methods section.

In the GLC cell experiment, results showed that insulin increased the effects of cisplatin, etopside, and 5-FU on the S phase arrest in GLC cells, with the enhancing effects being approximately 80%. Insulin increased the effect of 5-FU on the G_2/M phase arrest with an enhancing effect of approximately 90%. The increase of the cell cycle arrest indicated the increase of cytotoxic activity. The above results demonstrated that insulin had specific enhancing effects on selected chemotherapeutic drugs' cytotoxicity. When insulin was tested alone after 48 hr, the change in S phase was restored, suggesting the reversible effect of insulin. In the NEC cell experiment, insulin increased the effect of etopside on the G_2/M phase arrest in GLC cells, with enhancing effects of approximately 10-30%. Insulin increased the effects of 5-FU on the

G_2/M phase arrest with the enhancing effect of approximately 20%. Insulin did not have an obvious effect on cisplatin.

Discussion

The cytotoxicity of most chemotherapeutic drugs does not have specific selectivity; however, different cells show different outcomes in response to cytotoxic agents. Compared with normal cells, chemotherapeutic drugs have much stronger killing effects to cancer cells. Poorly differentiated, highly malignant tumors were more sensitive to chemotherapy. Gene states (or genotype) determine the level of differentiation of tumor cells, and ultimately determine the differences in metabolism and biological behavior. However, since gene defects and instability of tumor cells result in heterogeneity, a portion of tumor cells were able to survive after avoiding the cytotoxic attack by chemotherapeutic drugs. Thus, a combination therapy targeting gene level, cell behavior level, and metabolic level is an effective way to increase a chemotherapeutic drugs' sensitivity. Since poorly differentiated cells proliferate actively, they are susceptible to damage by chemotherapeutic drugs. Whether the sensitivity of chemotherapeutic drugs can be increased, by first using non-chemotherapeutic drugs to increase the metabolic level of tumor cells followed by chemotherapy. As an early step, data from this study approved this hypothesis.

1. Insulin as a reversible metabolic promoter: Insulin can promote cells to enter cell cycles and it can promote MTT metabolism, thus it is a metabolic promoter [2,3]. However, this study showed that when insulin was added it had a short-tem effect, but after 24-48 hr the cells metabolic changes returned to normal, which was a reversal process. Therefore, this change should not promote cell proliferation and increase malignancy for a long period. This short-term promoting effect of insulin would sometimes even shift into an inhibitory effect, probably due to the exhaustion of over metabolism. Thus, exogenous insulin should not induce the progression of malignant tumors, and it should be safe to use.

2. Different tumor cells have different responses to insulin: This study selected two cell lines that responded to the promoter similarly in general, but with different levels of sensitivity. The GLC (lung adenocarcinoma) cells were more sensitive to insulin's inducing effect. Thus, it is assumed that different individuals, different tumors, or the same tumor in different subpopulations, would have different responses to insulin, which would be related to factors such as cell surface receptor number, their distribution, functions, and so on. We are currently screening the enhancing effects of insulin and other metabolic promoters such as thyroid hormone on chemotherapeutic drugs' sensitivity in different tumor cell lines, to obtain supporting evidence for clinical practice.

3. The mechanism of insulin's promoting effect and its potential clinical application: By itself, or when it is combined with chemotherapeutic drugs, insulin increased cytotoxic effects of cisplatin, 5-FU, and etopside. The cell cycle theory is the basis for modern combined chemotherapy, and this theory is applicable to the combined use of promoter and chemotherapeutic drugs. Throughout this study, we tried to elucidate the general mechanism in which insulin promoted chemotherapeutic drug effects: after insulin addition, it acts on insulin receptors on the cells and protein D, etc. in the cell cycle are increased to promote more cells to enter the proliferating phase, and cell metabolic level is increased. At this point when chemotheraputic drugs are added, cells are more susceptible to injury, thus can increase the cytotoxic effect of chemotherapeutic drugs. From the experiment data, when insulin was added 8

hr before the chemotherapeutic drugs' addition, the enhancing effects could be seen, suggesting that the importance of drug addition was time-related. Insulin is a commonly used clinical medication and is safe at low-cost; thus, it is clinically practical to use insulin. When insulin was infused with suitable amounts of glucose (at routine dose), it has limited side effects. Furthermore, insulin is also used as a supportive therapy with practical value. The related clinical trial is currently being conducted. If the hypothesis that "increasing cell metabolism can increase chemotherapeutic drugs' effect" could be proven clinically, this would initiate a series of more effective promoters for chemotherapeutic drugs, and this approach should has a broad perspective in clinical medicine.

References:

1. Stone S, Dayananth P, Kamb A. Reversible, vp16-mediated cell cycle arrest as protection from chemotherapy. Cancer Res, 1996, 56: 3199-3202.

2. Cascinu S, Del Ferro E, Grianti C, et al. Inhibition of tumor cell kinetics and serum insulin growth factor I levels by octreotide in colorectal cancer patients. Gastroenterology, 1997,113:767-772.

3. Baserga R. The insulin-like growth factor I receptor: a key to tumor growth? Cancer Res, 1995, 55:249-252.

(Received for publication on November 19, 2002)

(Article Editor: Yuan Guiqing)