Glargine Promotes Human Colorectal Cancer Cell Proliferation via Upregulation of miR-95

Abstract

Several studies have shown a correlation between glargine use and cancer risk. However, the role of glargine in carcinogenesis, especially in colorectal cancer (CRC), is still inconclusive. The aim of this study was to investigate the influence of glargine on proliferation of CRC cells and its possible mechanism. Effect of glargine on the cell proliferation was tested in HCT-116 and SW480 cells by MTT assay, and apoptosis was measured by flow cytometry. The expression of micro-RNA-95 (miR-95) and sorting nexin 1 (SNX1) protein was also determined by real-time PCR and Western blotting, respectively. The results showed that high dose glargine (from 150 to 300 nM) promoted proliferation and inhibited apoptosis of CRC cells compared with untreated cells. Moreover, glargine could upregulate miR-95 and downregulate SNX1 protein expression in CRC cells. These data show that glargine may indeed trigger cellular proliferation in CRC, probably by regulating miR-95.

Introduction

Diabetes is a chronically progressive disease characterized by insulin deficiency. Exogenous insulin administration is the main treatment available for type 1 diabetes patients and is one of the main therapeutic approaches for type 2 diabetes patients. In order to improve the pharmacological properties of insulin, several insulin analogues have been developed by using DNA recombinant techniques and widely used for the treatment of diabetes mellitus [1, 2]. However, several epidemiological studies in 2009 raised the question of a possible relationship between the use of insulin analogues and cancer risk. These studies demonstrated that increased levels of exogenous insulin, particularly in the treatment with long-acting insulin analogue glargine, had experienced increased rates of colon, breast, and pancreatic cancers [3–5]. Since the publication of these studies, many subsequent studies, and meta-analyses have followed. Mannucci et al. [6] assessed the long-term association of different insulin analogues with cancer incidence. Consistent with previous researches, they found that incident cancer was associated with a higher daily dose of glargine, but had no association with human insulin or other analogues [6]. However, a growing body of recent epidemiological studies gave contradictory results indicating that there was no substantial evidence for increased risk of breast, prostate, colorectal, or other cancers combined associated with insulin glargine use [7–10]. Similarly, the lab researches regarding the effects of glargine on cancer were also inconsistent, particularly in CRC. Weinstein et al. demonstrated that insulin analogue glargine exhibited in vitro proliferative and anti-apoptotic activities in a number of cancer-derived cell lines, including CRC cells [11]. On the other hand, Nagel et al. observed no differences in epithelial colonic proliferation and aberrant crypt foci formation in glargine-treated mice compared with mice without glargine. They suggested that use of insulin glargine was not associated with increased risk of CRC [12]. Although many studies related to the oncogenic capability of glargine have been performed over several years, the relationship between glargine and cancer is still inconclusive. Thus, the role of glargine in carcinogenesis, especially in CRC, deserves further investigation.
rily binding to the mRNA 3’-untranslated regions (3’-UTR) in a sequence-specific manner, resulting in cleavage or translational repression of the target mRNA. Increasing evidences have shown that the deregulation of miRNAs is involved in a wide range of diseases, including human cancers. miRNAs might play important roles in biological processes that affect tumor progression including differentiation, proliferation, apoptosis, migration, and invasion [13,14]. However, until now very few researches have been done to elucidate the effects of glargine on miRNAs expression in carcinogenesis. The only study we could find that examined the alteration of miRNAs after glargine treatment detected ten miRNAs; especially miR-95, miR-134, and miR-34c-3p, were significantly upregulated by glargine in human pancreatic cancer cells [15]. Moreover, miR-95 was reported to be frequently upregulated in various malignancies, including colorectal cancer [16], breast cancer [17], prostate cancer [17], pancreatic cancer [15], and nonsmall cell lung cancer (NSCLC) [18]. And SNX1, a putative tumor suppressor of CRC [19], was demonstrated as a direct functional target of miR-95 [16,18]. This suggested that miR-95 might act as an important oncogene by negatively regulating SNX1. Therefore, modulation of miR-95 expression may be an important mechanism underlying the promotion effects of glargine on CRC progress. However, no studies have been reported on this aspect.

Thus, in the present study, we sought to investigate the influence of glargine on proliferation, apoptosis, and miR-95 expression in CRC cells, preliminarily revealing the possible molecule mechanism of the biological actions of glargine.

Materials and Methods

Cell culture and glargine treatment

The human CRC cell lines HCT-116 and SW480 were obtained from Shanghai Institute of Cell Biology. Cells were cultured in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, and kept at 37 °C in a humified 5% CO2 incubator. For glargine treatment, media was replaced with fresh serum-free medium containing glargine, when cells were grown to 60% confluency. All cells were starved with low-serum medium (containing 0.5% FBS) for 24h before the experiments.

Cell proliferation assay

Effect of glargine on proliferation was assessed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, CRC cells were plated at a density of 4 × 104 cells/well into 96-well plates. After overnight incubation, cells were treated with glargine in different concentrations ranging from 10 to 300 nM for 48h. At the end of the treatment, 20μl MTT (5mg/ml) was added to each well and incubated for additional 4h. Then, the medium was removed, and 150μl DMSO was added to each well to dissolve the sediment. The optical density (OD) for each well was determined at 570nm on a microplate reader (Bio-Rad, USA) after shaking for 10min at room temperature.

Flow cytometry

The cell apoptosis was evaluated by flow cytometry analysis. After treatment with glargine for 48h, cells were harvested and dual stained with Annexin V-FITC and propidium iodide (PI) using an apoptosis detection kit (KaiJi, China) following the manufacturer’s protocol. Stained cells were immediately analyzed by the flow cytometry (Becton Dickinson, USA). Viable cells are primarily Annexin V and PI negative (PI positive staining indicates necrosis), and only cells stained with Annexin V were considered to undergo early-stage apoptosis.

RNA isolation and real-time PCR

CRC cells were treated with glargine for 48h, then the total RNA was isolated from cells using Trizol reagent (Invitrogen, USA) according to manufacturer’s instructions. Then, cDNA synthesis was performed using the RevertAid™ First Strand cDNA Synthesis kit (Fermentas, USA). For detection of miR-95 expression, stem-loop RT-PCR was performed as previously described [20]. Real-time PCR was carried out using SYBR Premix Ex Taq™ (Takara, Japan) according to the manufacturer’s protocol. U6 small nuclear RNA (U6) served as the endogenous control. The primers for miR-95: stem-loop RT primer: 5’-CTCAACTGTTGCTGTTGAGCTCGCAAATTCAGGAGCTCATT-3’; forward: 5’-CCGGTATTATTTACGACCA-3’ and reverse: 5’-AACGGTCTGGTTCGCAG-3’. The primers for U6: RT primer: 5’-CGCTTCAGAATTCCGTCGTAAT-3’; forward: 5’-CTCCCTTGGCCAGCACA-3’ and reverse: 5’-AAGCCTTACCAAGATTTGG-3’. The relative expression was calculated using the 2^-ΔΔCt method.

Western blot analysis

After treating with different concentrations of glargine for 48h, the cells were harvested with ice-cold PBS and lysed on ice in lysis buffer for 30min. The lysates were centrifuged 1.2 × 10^6 rpm for 10min, and the resulting supernatants were determined by Bio-Rad protein assay kit (Bio-Rad, USA) using bovine serum albumin as the standard. Equal amounts of proteins were electrophoresed by SDS-PAGE gels and subsequently transferred to PVDF membranes (Bio-Rad, USA). Membranes were blocked with 5% nonfat dried milk in TBST (Tris-buffered solution, pH 7.6, 0.05 % Tween 20) and then incubated with mouse anti-SNX1 (1:400, Abcam, UK), and mouse anti-β-actin (1:1000, Santa cruz, USA) at room temperature for 2h. After 3 washes, the blots were subsequently incubated with appropriate secondary antibodies (1:2000, Santa Cruz, USA) coupled to horseradish peroxidase at room temperature for 1h, and developed in the ECL Western detection reagents (Beyotime, China). Expression levels of the proteins were compared to the control based on the relative intensities of the bands. Band density was quantified using the software program Bio-Rad Quantity One v4.62.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) as appropriate. All statistics and data analysis were performed using SPSS 13.0 software (SPSS Inc., USA). Data are presented as means ± standard deviation (SD) A value of p < 0.05 was considered statistically significant.

Results

Glargine promotes proliferation of CRC cells

We first examined how the treatment with glargine for 48h influenced proliferation of HCT-116 and SW480 cells. The MTT assay results are shown in Fig. 1. Glargine dramatically increased the cell number of HCT-116 and SW480 cells at higher concentrations (from 150 to 300nM) as compared with untreated cells (p < 0.05). However, the low concentrations of
glargine (from 10 to 100 nM) exhibited no proliferative effect on both HCT-116 and SW480 cells (p > 0.05).

Glargine regulates apoptosis of CRC cells
After 48 h incubation of HCT-116 and SW480 cells with different concentrations of glargine, flow cytometric analysis was performed to determine cell apoptosis. As shown in Fig. 2, the untreated group showed 19.81% and 25.33% apoptosis rate in HCT-116 and SW480 cells, respectively. However, after treatment with glargine (150, 200, and 300 nM) for 48 h, the apoptosis percentage decreased to 14.93%, 12.40%, 8.67% in HCT-116 cells, and 20.47%, 15.87%, 12.94% in SW480 cells, respectively. The result showed that glargine might effectively inhibit apoptosis of CRC cells in a dose-dependent manner.

Glargine upregulates the expression of miR-95
To study the regulation of glargine on miR-95, stem-loop real-time PCR was performed. Compared with untreated cells, the expression of miR-95 was upregulated by 1.71-fold (p < 0.05), 2.47-fold (p < 0.01), and 3.17-fold (p < 0.01) on average, respectively, in HCT-116 cells (Fig. 3a), and 1.53-fold (p < 0.05), 2.73-fold (p < 0.01) and 3.09-fold (p < 0.01) on average, respectively, in SW480 cells (Fig. 3b), after treatment with increasing concentrations of glargine (150, 200, and 300 nM) for 48 h. The data demonstrated that glargine might upregulate the expression of miR-95 in a dose-dependent manner in CRC cells.

Glargine downregulates the protein expression of SNX1
Western blot analysis was performed to examine SNX1 protein expression (the direct target of miR-95) in CRC cells under glargine treatment. As shown in Fig. 4, the protein level of SNX1 in both HCT-116 and SW480 cells was downregulated after incubation with glargine for 48 h, especially in the 200 nM and 300 nM glargine-treated group as compared with untreated cells (p < 0.01).
Discussion and Conclusion

Glargine (A21Gly, B31Arg, B32Arg human insulin), which differs from human insulin by adding 2 arginine molecules at the C-terminal region of the insulin B chain (position B30), and the substitution of glycine for asparagine in the A chain (position A21), is widely used as a long-acting insulin analogue in the treatment of diabetes mellitus [21]. Previous experimental studies have shown that glargine may have potential carcinogenic effects [3–6,11]. Thus, Weinstein et al. found that glargine could display IGF-I-like proliferative and anti-apoptotic activities in colorectal (HCT-116), prostate (PC-3), and breast (MCF-7) cancer cells [11]. Furthermore, Liu et al. demonstrated that high dose insulin glargine could promote T24 bladder cancer cell proliferation via PI3K-independent activation of Akt [22]. Similarly, in the breast adenocarcinoma cells, glargine also exhibited stimulatory effects on the proliferation involved in activation of Akt and regulation of apoptosis-related gene bax/bcl-2 [23]. However, other studies gave contradictory results, which showed that the glargine might be unrelated to the occurrence and development of tumors, particularly in CRC. Karlstad et al. [9] performed a systematic review and meta-analysis of published cohort and case-control studies to assess the association of insulin use and cancer risk. While they found no substantial evidence for increase in CRC risk among insulin glargine users as compared to non-glargine users, the glargine users did show an increased risk for breast cancer [9]. Nagel et al. also demonstrated that glargine had no effects on the tumorigenesis of CRC in diabetic mice [12]. In the present study, we detected the in vitro biological effects of glargine in CRC cell lines. Results of proliferation and apoptosis assays showed that high dose glargine could significantly promote proliferation and inhibit apoptosis of HCT-116 and SW480 cells, which was reported as a putative tumor suppressor involved in cell proliferation and apoptosis in CRC [19]. In addition, Wei et al. found that high dosages of glargine could significantly upregulate 10 miRNAs (miR-665, miR-720, miR-134, miR-486–5P, miR-570, miR-545, miR-501–5P, miR-34c–3P, and miR-95), especially miR-95, miR-134, and miR-34c–3p, and downregulate 2 miRNAs (miR-105 and miR-181a) in human pancreatic cancer cells [15]. In the present study, we elucidated the alteration of miR-95, a potential oncogene in CRC, in response to glargine treatment in human CRC cells for the first time. In agreement with previous reports, our findings have shown that miR-95 was significantly elevated in high dose glargine-treated CRC cells. Moreover, SNX1, the target of miR-95, was also downregulated by high dose glargine. These data suggested that upregulation of miR-95 and subsequently inhibition of SNX1 protein might be associated with the proliferative and anti-apoptotic effects of glargine in CRC.

In summary, the current results demonstrate that glargine exhibits in vitro proliferative and anti-apoptotic activities in CRC cells, probably by upregulating miR-95. It provides a novel evidence for the relationship between glargine and CRC. However, further studies are required to evaluate the in vivo effects and molecular mechanisms of glargine in CRC.

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Declaration of Interest

The authors have no conflicts of interest.

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Fig. 4 Effect of glargine on SNX1 protein expression in CRC cells. HCT-116 and SW480 cells were incubated with increasing concentrations of glargine (150–300 nM) for 48 h. Then, the protein expression of SNX1 was detected by Western blot analysis. The results were normalized to β-actin protein expression. a The relative protein expression of SNX1 in HCT-116 cells, and b in SW480 cells. All data are representative of 3 independent experiments. * p<0.05, ** p<0.01 vs. untreated cells.
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