**BACKGROUND**

Hypoxia and the resultant oxidative stress play an important role in the development, incidence and severity of post-operative adhesions. The inflammatory response to injury, such as hypoxia sustained during surgical procedures, involves a complex cascade of events orchestrated, at least in part, by hypoxia inducible factor (HIF)-1α. Among the key events in the cascade are induction of transforming growth factor-β1 (TGF-β1) cellular and vascular endothelial growth factor (VEGF), and development of the adhesion phenotype in peritoneal fibroblasts, all of which promote the profibrotic inflammatory response. Fibron and collagen deposition at the wound site, as part of the hypoxia response, helps to establish a condition for peritoneal wound healing. When such processes proceed unchecked, as in the fibrotic state, peritoneal adhesion formation results.

L-Alanyl-L-Glutamine (Ala-Gln) is a dipeptide comprised of two amino acids, alanine and glutamine, which are conditionally essential. Glutamine’s known biological effects suggest a potential role for Ala-Gln in preventing postsurgical adhesions by modulating early events in wound healing and the cellular response to hypoxia.

**OBJECTIVES**

To determine the effect of L-Alanyl-L-Glutamine on HIF-1α and type I collagen levels in normal human peritoneal fibroblasts under conditions of continuous or episodic hypoxia, compared to prehypoxic or untreated controls.

**METHODS**

**Source and Culture of Human Fibroblasts:** Normal parietal peritoneal tissue from the anterior abdominal wall lateral to midline incision was excised from patients undergoing laparotomy for pelvic pain, at the initiation of the surgery following entry into the abdominal cavity. Normal peritoneum was at minimum three inches from any adhesions. Subjects did not have an active pelvic infection and were not pregnant. All patients gave informed written consent to tissue collection, which was conducted under a protocol approved by the Wayne State University Institutional Review Board. Harvested tissue specimens from five women were immediately placed in standard media (DMEM containing 10% fetal bovine serum, 2% penicillin and streptomycin). Tissues were cut into small pieces in a sterile culture dish and transferred into another fresh T-25 flask with 3 ml of displace solution (2.4 U/l, Gibco BRL. Life Technologies, Inc.). The samples were then centrifuged for min, 200 g. Extracted tissue was then transferred to a fresh T-25 flask with pre-warmed DMEM medium, and placed in 37°C incubator (95% air and 5% CO2) outgrowth of fibroblasts generally took 2 weeks. Once confluence was reached, the cells were transferred to 90-mm tissue culture dishes and cultured in standard media with 10% FBS. Thereafter, the confluent dishes were subcultured by trypsinization (1:3 split ratio). All experiments were performed in triplicate.

**Hypoxia Treatment:** All hypoxic experiments were performed in an airtight modular incubator chamber (Billups-Rothenberg). The chamber was deoxygenated by a positive infusion of 2% O2 in a CO2-nitrogen gas mixture. In Case 1 (continuous exposure to hypoxia), normal peritoneal fibroblasts cells (1.5 x 10⁶) were cultured in 60 mm² tissue culture dishes under hypoxic (2% O2) conditions for 0, 0.5, 1, 2, 4, 12, 24, and 48 hours in a 37ºC incubator with or without Ala-Gln (1, 2, 10 mM and no restoration of normoxia). Cells were collected at each indicated observation point. The sampling times all occurred after normoxia was restored, however, they were calculated from the start of the experiment, which was the start of hypoxia exposure.

**ELISA:** Cell pellets were resuspended in PBS and sonicated on ice for protein extraction. Total protein concentration of cell lysates was measured with the Pierce BCA Protein Assay Kit (Thermo Scientific) per the manufacturer’s protocol. Equal concentration of protein was utilized in each ELISA. Protein was stored at -80°C and was thawed on ice before use in the ELISAs. Levels of HIF-1α and type I collagen levels were determined utilizing the ELISA kit (ThermoFisher Scientific) or the COL1A1 ELISA (Lifespan Biosciences), respectively, per the manufacturer’s protocol.

**Statistical Analysis:** Data were analyzed using SPSS 23.0 for Mac. Pair-wise differences in normally distributed variables in treatment and control groups were compared using one-way ANOVA with significant interactions further analyzed using Tukey post hoc analysis with Bonferroni correction.

**RESULTS**

Levels of both HIF-1α and type I collagen increased in untreated controls by about 3 to 4-fold in response to continuous as well as episodic hypoxia, in normal human peritoneal fibroblasts. Under continuous hypoxia, the hypoxia induced increase in HIF-1α and type I collagen was suppressed dose dependently upon treatment with Ala-Gln (Figure 1). Episodic hypoxia (0.5, 1, 2 or 4 hours) followed by restoration of normoxia and immediate treatment with Ala-Gln, resulted in consistent suppression of induced levels of HIF-1α (hypoxic episode of 2 hours or less) and type I collagen (regardless of hypoxic episode), for up to 24 hours sampling time at all doses, and up to 48 hours sampling at the highest dose (Figures 2 & 3).

**CONCLUSIONS**

L-Alanyl-L-Glutamine significantly and persistently suppressed hypoxia-induced levels of key adhesion phenotype markers, HIF1α and type 1 collagen, under conditions of as well as episodic hypoxia in normal peritoneal fibroblasts, in vitro.

Ala-Gln suppression of hypoxic induction of HIF-1α, a known trigger of the inflammatory response that culminates in fibrosis, suggests potential therapeutic value for the dipeptide in the prevention of postoperative adhesions.