Involvement of p21<sup>cip1/waf1</sup> in the anti-proliferative effects of polyethylene glycol in colon carcinogenesis

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Abstract. Polyethylene glycol (PEG) is a safe and effective chemopreventive agent against colorectal carcinogenesis in cell culture, animal models and human subjects. Although the precise molecular mechanism is unclear, we previously reported that PEG suppresses colonic epithelial proliferation. As cellular proliferation is driven by complex G1-S phase transition, we now characterize the role of PEG on cell cycle regulation. We focused our attention on the effect of PEG on the CDK inhibitor p21<sup>cip1/waf1</sup>, which is implicated in early colon carcinogenesis and is upregulated by non-steroidal anti-inflammatory drugs. These studies were done in the azoxymethane-treated (AOM) rat model as well as in HT-29 colon cancer cells. Immunohistochemical analysis revealed that while AOM decreased the p21 expression (75%, p<0.01) in the premalignant colonic mucosa, PEG induced p21 levels back to normal. These findings paralleled a decreased BrdUrd incorporation (78%, p<0.001) and hypophosphorylated retinoblastoma protein (Rb; by 47%) signifying PEG's antiproliferative activity. Furthermore, in HT-29 cells, PEG decreased proliferation as measured by PCNA (68% reduction), increased p21 expression (2.3-fold), induced cell cycle arrest during G0/G1 phase (45% reduction) and neutralized the phosphorylation of Rb (by 52% compared to untreated). PEG caused greater than a 2-fold induction of protein and mRNA level of p21<sup>cip1/waf1</sup> in HT-29 cells. These results demonstrate for the first time that PEG is involved in p21 regulation concomitant with G1-S phase cell cycle arrest and it is through these effects that it can exert its anti-proliferative and hence chemopreventive role.

Introduction

Colorectal carcinogenesis involves a sequential accumulation of molecular events that drive morphological progression in colon carcinogenesis (adenoma-carcinoma sequence). Even prior to microscopic alterations (dysplasia), there are profound genetic/epigenetic changes that have consequences for epithelial cellular homeostasis providing the underpinnings for tumorigenesis. These molecular alterations are symbolized by increased cellular proliferation rate diffusely in the histologically-normal epithelium that may be linked to decreased apoptosis. From a teleological perspective, this diffuse mucosal hyperproliferative milieu allows the initiated clones the opportunity to rapidly expand and develop distinct morphological lesions. This cellular growth augmentation has several potential applications such as providing a marker of field carcinogenesis and a possible target for cancer prevention. As a specific example, epithelial proliferation rates in the rectum have been shown to be robust markers of field carcinogenesis (1) that has promising relevance in chemoprevention validation.

There have been a myriad of agents purported to have chemopreventive efficacy against colon carcinogenesis. The best studied are the non-steroidal anti-inflammatory drugs (NSAIDs) with other putative agents including calcium, folate, ursodeoxycholic acid and vitamin D (2-5). All of these have been shown to decrease colorectal cancer (CRC) risk in experimental as well as clinical studies. While numerous mechanisms have been implicated, reversal of the colonic epithelial hyperproliferation appears to be of major importance. Indeed, there is strong evidence that for many agents, proliferation in the uninvolved mucosa tends to be a reliable intermediate biomarker for chemoprevention (3,6-9). Unfortunately, none of the well-established anti-proliferative agents could be exploited in long-term clinical practice mainly over concerns of marginal efficacy (calcium, folate, fiber) and above all toxicity (higher gastrointestinal or cardiovascular toxicity for NSAIDs) (10,11). Thus, novel agents with greater efficacy and lower toxicity profile are urgently warranted in any significant clinical setting. Polyethylene glycol (PEG) has recently received attention as a promising chemopreventive agent of a superior capacity with potent anti-proliferative properties in both cell culture and animal models of colon carcinogenesis (12-14). This promising effect has also been corroborated in a pilot clinical (case-control) study (15). The chemopreventive efficacy of PEG appears to be better than that of widely used NSAIDs (16) and notably, its safety profile appears outstanding given that it is not systemically absorbed. Indeed, PEG is available in most countries as a major component of an over-the-counter laxative (17).
To further develop PEG as a chemopreventive agent, a central question is to better understand the molecular mechanism of inhibition of proliferation and hence colon carcinogenesis. The regulation of colonocyte proliferation is governed by complex molecular interactions between various cyclins, cyclin dependent kinase (CDKs) and CDK inhibitors (CDKIs). By in large, the most critical event appears to be modulation of the G1-S phase directed through these factors. Recent attention has focused on CDK inhibitors such as p21\textsuperscript{cip1/waf1}, p16\textsuperscript{INK4a} and p27\textsuperscript{kip1} (18). These bind CDKs and prevent phosphorylation of the retinoblastoma (pRb) tumor suppressor gene with consequent silencing of the translation factor E2F1. Most of these CDK inhibitors are implicated in early colon carcinogenesis, however, for chemoprevention particular attention has been focused on p21 because of being upregulated by agents such as NSAIDs in wild-type mice with loss of response to these agents in transgenic p21 knockout mice (19). Thus, p21\textsuperscript{cip1/waf1} would appear to be a promising target for PEG antiproliferation, although this area of investigation has been largely unexplored.

In this report, we demonstrate that in the well-validated azoxymethane (AOM)-treated rat model, p21\textsuperscript{cip1/waf1} is lost in early colon carcinogenesis. However, the oral administration of PEG reverts the levels back to normal. These changes were mirrored by reduction in colonic epithelial proliferation as a result of PEG exposure. We then performed cell culture studies which mirrored the PEG-mediated p21\textsuperscript{cip1/waf1} induction in standard cell lines with a G1-S phase arrest, supporting the paramount function of p21\textsuperscript{cip1/waf1} in PEG-mediated chemoprevention.

Materials and methods

Experimental animal protocols. Male Fisher 344 rats (125-150 g; 4-5 weeks of age) were procured (Harlan Laboratories, Indianapolis, IN) and all the animal studies were conducted in accordance with the Institutional Animal Care and Use Committee of Northshore University HealthSystems. The rats were maintained on a defined (AIN-76A) diet for one week before randomized into three equal groups. The rats in groups 1 and 2 received intra-peritoneal injections of AOM (Sigma Chemicals, St. Louis; 15 mg/kg body weight/week for 2 weeks), while group 3 rats received equivalent volume of saline as vehicle. Two-weeks post AOM, group 2 rats were switched to a PEG-8000 supplemented diet (10 g/100 g; Harlan Teklad) and continued for another 10 weeks until sacrifice. Rats were housed in polycarbonate cages in a climate controlled room and provided clean water \textit{ad libitum} (via in-house automatic watering system). Rats were routinely sacrificed mid morning (to control for diurnal variations) in a non-fasted state for ~10 h 2 h after the administration of BrdUrd (i.p.; 50 mg/kg body weight) to label cells \textit{in vivo} in the S-phase. Colons were flushed with cold saline and small distal segments were fixed in buffered formalin and prepared for immunohistochemical studies.

Immunohistochemical (IHC) analysis. IHC staining technique was used to assess changes in the expression of p21 and the rate of cellular proliferation as quantified by BrdUrd staining. For this analysis, 4 micron sections were sliced from the paraffin-embedded tissue samples and mounted on Superfrost Plus glass slides (Vector Laboratories, Burlingame, CA). The slides were heated at 60°C for 1 h, de-paraffinized by two xylene washes and then hydrated in graded series of ethanol washes (70%, 95% and absolute). The antigen retrieval was achieved by pressure microwaving (NordicWare, Minneapolis, MN) the slides for 15 min in antigen unmasking solution (Vector Laboratories). Endogenous peroxidase activity was quenched by immersing the slides in 3% H\textsubscript{2}O\textsubscript{2}/methanol solution for 10 min and non-specific binding was blocked by 5% horse serum for 1 h (Vectastain Elite ABC kit; Vector Laboratories) at room temperature. Sections were then incubated overnight with primary antibodies anti-p21\textsuperscript{cip1/waf1} (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-BrdUrd (1:50; Zymed Laboratories, Inc., San Francisco, CA), followed by 1 h of incubation with the appropriate biotinylated secondary antibodies. After repeated washing, the sections were developed using ABC reagents from the kit. Only complete longitudinal crypts extending from the muscularis mucosa to colonic lumen were considered for immunohistochemical evaluation (10 crypts in each colon and 8 rats in each group).

Cell culture and PEG treatment. The human colon cancer cell line HT-29 (American Type Culture Collection) was cultured in McCoy’s 5A medium with 10% fetal bovine serum. The cells were seeded in 100 mm Petri dishes (10\textsuperscript{4} cells/ml), washed twice with PBS, and serum starved (0.5% fetal bovine serum) for 72 h before treating with PEG for 24 h. Cells were then harvested and subjected to flow cytometric analysis, Western blotting or reverse transcription-PCR.

SDS gel electrophoresis and Western blot analysis. Equal amounts of protein (25 μg) from cell lysates (suspended in Laemmli sample buffer) were separated by SDS-PAGE and transferred onto Immobilon-P transfer membranes (Millipore Corp., Bedford, MA). Immunoblots using the appropriate antibody was then developed using standard techniques. Xerograms were created with enhanced chemiluminescence (Santa Cruz Biotechnology) and image analysis was done using image acquisition analysis software (Labworks, 4.6; UVP). Expression levels were normalized to the levels of β-actin. To enhance the detection level of p21, the lysates were first subjected to p21 immunoprecipitation. For this, the cell extracts were precleared with protein G-agarose beads for 2 h at 4°C, and then further incubated with 2 μg p21 antibody and beads with constant shaking on a rocking platform. The immunocomplexes were pelleted and washed 3 times with the lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 15 mM MgCl\textsubscript{2}, 5 mM EDTA, and 1% NP-40 with protease inhibitor cocktail). The immunocomplexes and the cell lysates were then run on SDS-PAGE as above.

RT-PCR analysis. HT-29 cells were treated with 10% PEG-3350 or PEG-8000 for 24 h and total RNA was isolated using TRI-Reagent as recommended by the manufacturer (Sigma Chemical Co.). The cDNA was amplified from 2 μg of total RNA using a SuperScript -Taq system (Invitrogen Life Technologies). PCR was conducted for 22-30 cycles in a thermal cycler (Eppendorf, Master Cycler Gradient). Primers
used for amplification were as follows: p21-specific primer set, p21F (5'-GTG AGC GAT GGA ACT TCG A-3') and p21R (5'-AAT CTG TCA TGC TGG TCT GC-3'); ß-actin-specific primer set, ßAF (5'-GGC ATC GTG ATG GAC TCC G-3') and ßAR (5'-GCT GGA AGG TGG ACA GCG A-3'). ß-actin specific PCR products from the same RNA samples were amplified to serve as internal loading controls.

Propidium iodide (PI) staining and flow cytometric cell cycle analysis. HT29 cells were seeded into 6-well plates (1x10^5 cells per well), serum starved, treated with 10% PEG (PEG-3350 and PEG-8000 separately) or vehicle for 24 h. The cells were then trypsinized and fixed overnight in chilled 70% EtOH (-20°C). The cells were stained in propidium iodide (50 μg/ml for 3 h at room temperature) and DNA content measured by flow cytometric analysis (Becton-Dickinson Labware) using CellQuest 3.1 software for data analysis. Experiments were done in triplicate.

EGFR knockdown assay. The EGFR gene expression was knocked down in HT-29 cells using shRNA (Origene) as described previously (13). The lysates were then subjected to Western blotting to assess p21 expression.

Statistical analysis. Values were expressed as mean ± SD as indicated. For statistical analysis, unpaired Student’s t-test was used and between the groups; a p<0.05 was considered significant.

Results

PEG stimulates colonic epithelial p21 expression in AOM-induced rats. Our group has previously shown that PEG treatment suppresses epithelial proliferation in the well-validated AOM-treated rat model (12). Because p21 is known to be an important regulator of cell cycle which is lost in early colon carcinogenesis (20), we assessed the effect of PEG treatment on this cell cycle regulator in the AOM-treated rat model. The rats were maintained on either AIN-76A diet or PEG-supplemented diet as described in ‘Materials and methods’. After 8 weeks of diet initiation, the rats were euthanized 2 h after BrdUrd injection (50 mg/kg, IP). Distal colon segments were fixed in formalin for 4 h and then transferred to 70% alcohol before sectioning and mounting on the glass slides. The rate of epithelial proliferation was measured by detecting BrdUrd incorporation using a detection kit (Zymed Laboratories). Panel A, representative immunohistochemical staining of the BrdUrd from the indicated groups (saline– AOM- and AOM treated rats) on AIN-76A diet supplemented with PEG-8000, 10 g/100 g diet (n=8 in each group). Note that crypts from AOM-PEG group have reduced BrdUrd staining and increased expression of cell cycle marker p21. On comparing groups (saline vs. AOM, filled star) or (AOM vs. AOM +PEG, filled circle), the changes for both p21 and BrdUrd labeling (Panel B) were found to highly significant (p<0.001).
euthanized and distal colonic segments formalin-fixed and prepared for IHC staining. As shown (Fig. 1) and consistent with our earlier report (12), AOM resulted in increased colonic epithelial proliferation compared to saline injected rats, as measured by epithelial BrdUrd incorporation. On the other hand PEG supplementation markedly reduced the BrdUrd incorporation in the AOM rats (by ~70%). When the tissue sections from these groups were immunostained for p21 expression, we noted that colonic sections from AOM-PEG group that expressed reduced BrdUrd staining also had a corresponding augmentation in the expression of cell cycle marker p21. These findings reveal contrasting effects of PEG i.e. reduced epithelial proliferation and increased p21 expression in the AOM-rat model of colon carcinogenesis.

PEG causes HT-29 cells to arrest in the G0/G1 phase of the cell cycle. Cell cycle analysis was performed to examine whether PEG-treated cells arrest in a specific phase of the cell cycle. Flow cytometric analysis indicated that when HT-29 cells were treated with 10% PEG-8000, the cells underwent a distinct increase in the G0/G1 population with a corresponding decrease in S-phase. After 24 h treatment with PEG, the relative percentage of cells in the G1 phase increased by 14% after 24 h and this was associated with a concomitant decrease of cells in the S-phase (44.4%) of the cell cycle (Fig. 2). From these results, it is clear that cell cycle arrest in the G1 phase may contribute to the anti-proliferation effect of PEG.

Treatment of HT-29 cells with PEG induces p21<sup>cip1/waf1</sup> and inhibits PCNA incorporation and phosphorylation of retinoblastoma protein (Rb). To complement the effect of PEG in cell cycle arrest, we studied the effect of PEG on the proliferative index, as measured by PCNA incorporation, and found that PEG suppressed the cellular proliferation by about 80%. As we found that PEG decreased cellular proliferation and induced G1 arrest in the cell cycle and inhibited cyclin D1 (our previous data) (12), Western blot analysis was performed to determine whether treatment of HT-29 cells with PEG alters cellular levels of cell cycle protein inhibitor...
Our results show that PEG dramatically induced the protein expression levels of CDK inhibitor, p21cip1/waf1 (Fig. 3). In view of this finding that PEG augmented cellular levels of p21cip1/waf1 protein, we examined whether this was associated with increased levels of p21cip1/waf1 mRNA expression by using a semiquantitative RT-PCR assay. Our results show that PEG caused more than 2-fold increase in the p21 message (Fig. 3).

The retinoblastoma tumor suppressor protein, Rb, in association with cyclin D and CDKs, acts as a critical regulator for the G1-S phase cell cycle progression. We tested the possibility that PEG causes cell cycle arrest through dephosphorylation of Rb. As shown PEG decreased phosphorylation at Ser795 of Rb by 52% (p<0.01) in HT-29 cells (Panel A) and 47% (p<0.05) in AOM-treated rats (Panel B), which suggests that cell cycle progression from G1 to S is inhibited by PEG.

PEG's effect on p21 may be mediated via its effect on EGFR. Consonant with our earlier findings, we found that PEG caused a significant decrease (p<0.01) in the EGFR expression (Panel A). We also found that p21 expression was higher (104%; p<0.01) in EGFR knock down HT-29 cells (shRNA-EGFR) compared to wild-type cells (Panel B). These results imply that EGFR may be an upstream mediator of the PEG effect on p21 expression.

**Discussion**

We show herein, for the first time, that PEG treatment induced p21 expression in the premalignant colonic mucosa of AOM-treated rats and these findings were mirrored by this potent chemopreventive agent's anti-proliferative activity. Our novel observation that PEG induced a G1-S phase arrest which was consonant with a significant role for p21cip1/waf1 provides an important mechanistic lead in PEG-mediated chemoprevention.
PEG is a remarkable chemopreventive agent whose efficacy was first reported by Corpet and colleagues using the AOM-treated rat model (14,21,22). They noted a rapid, dose-dependent reduction in both aberrant crypt foci (ACF) and tumors. The efficacy of PEG was truly remarkable, resulting in a 90% reduction in tumors. These findings were corroborated by our group (12). To keep these results in perspective, PEG outperformed all other chemopreventive agents including well established agents such as NSAIDs. Our group confirmed that this was not model-specific by demonstrating the efficacy of PEG 3350 in the MIN (multiple intestinal neoplasia) mouse model, a genetically driven model of intestinal neoplasia (23). While definitive (randomized placebo-controlled) studies are ongoing, it is heartening to note a 52% neoplasia risk reduction with PEG in a case-control study (15). The effect size in this clinical study is at least comparable and probably superior to the efficacy reported with NSAIDs.

Aside from enhanced efficacy as a chemopreventive agent, the other major advantage of PEG is its low toxicity resulting in an extremely favorable risk-to-benefit relationship. This is critical because CRC has a relatively low prevalence in the population, despite being the second leading cause of cancer deaths among Americans. Indeed, the lifetime risk of CRC is about ~5.5% which means that majority of the patients treated with a chemopreventive agent would most likely never develop CRC. Thus, due to the comparatively low incidence of this disease, the potential harm of this agent should be minimal to make a large scale population intervention feasible. Even though aspirin, which has a relatively low toxicity (mainly gastrointestinal), was not recommended for population intervention by the US Preventive Services Task Force because of the less than favorable harm-benefit calculation (24). Attempts to mitigate the GI side effects by using cyclooxygenase (COX)-2 specific NSAIDs however, resulted in severe cardiac toxicity. Even though these cardiac events had lower absolute rates (~5%), they resulted in much higher prevalence of deaths from cardiovascular disease versus that by CRC, making this approach untenable (10,11). Of some concern are recent reports that the cardiovascular toxicity may also be seen in non-COX-2 selective agents (i.e. sulindac) (25). Interventions such as calcium have been proven very safe but may have minimal efficacy (26). PEG, on the other hand, is not only very efficacious but remarkably non-toxic (27). This is largely due to the fact that it is not systemically absorbed. The only major side effect relates to hyperosmotic effect in the GI tract (diarrhea, bloating). Fortunately, studies in the AOM-treated rat showed efficacy without any obvious diarrhea. Moreover, given that 20-30% of the American population is constipated, some pro-motility effects may not be insurmountable.

Understanding the mechanism of action of PEG would be important in fostering translation of PEG's chemopreventive activity into the clinical arena by both supporting its biological plausibility and also enabling the potential of rationally-designed PEG formulations that would maximize efficacy and/or minimize side effects. In the studies on AOM-induced ACF suppression, the optimal formulation of PEG has been shown to be PEG-8000, though understanding its molecular targets may help redefine the specificity and effectiveness of other formulations. Previous reports on PEG's potential mechanism of action have suggested number of possibilities i.e., suppression of proliferation, induction of apoptosis, epitheliolysis (28,29). While these and other mechanisms are likely to contribute to the overall chemopreventive efficacy, the data supporting the importance of suppression of proliferation in chemoprevention is unequivocal. This may relate to the primary significance of increased proliferation in early colon carcinogenesis. Indeed, the diffuse hyperproliferation associated with colon carcinogenesis has been shown to reliably mirror the presence of neoplasia (30). The corollary is that suppression of proliferation by chemopreventive agents have been proven to be a reasonable measure of long-term efficacy (30). This has been demonstrated for a variety of agents including aspirin and calcium. Thus, the antiproliferative effects of PEG seem to be inextricably related to chemoprevention.

The ability of PEG to target some of the earliest events in colon carcinogenesis such as proliferation is consonant with its potent inhibition of the full spectrum of neoplastic lesions (ACF, adenomas and cancer) in experimental animal study reports (12,14). These have been complemented by reports that PEG has inhibited proliferation in a wide variety of CRC cell lines (12,13,29) although, the mechanisms had remained largely unexplored. Thus, our data demonstrated that there was a striking inhibition of G1-S phase progression suggesting that this was a key factor in the suppression of proliferation by PEG. Further supporting the importance of this finding is that G1-S blocks are seen with a variety of chemopreventive agents against CRC including NSAIDs, resveratrol. This provided the rationale to focus our efforts on elucidating the effect of PEG on this critical cellular process.

The molecular interplay that governs the G1-S phase checkpoint of the cell cycle is multifaceted. During colon carcinogenesis, much attention has been focused on the events related to dysregulation of β-catenin signaling, the putative initiating event in most colonic neoplasia. β-catenin transcriptionally regulates a variety of proteins including cyclin D1, which is an important positive regulator of G1 to S cell cycle transition and upregulated in colon cancer. We have previously reported that PEG inhibits cyclin D1 expression (12). In general, most studies with chemopreventive agents have targeted pro-proliferative cyclins, however, recently several lines of evidence suggest that modulation of cyclin-dependent kinase inhibitors may also be involved. In particular, p21 induction has been seen in colon cancer cell lines with a myriad of agents ranging from NSAIDs, curcumin and resveratrol. Thus, our observation that PEG causes a striking upregulation in p21 protein and message may provide another potential mechanism for PEG-induced cell cycle arrest.

The fundamental question is whether induction of p21 is important in chemoprevention or simply a bystander. For p21 regulation by PEG to be relevant in chemoprevention, it would need to be an early event in colon carcinogenesis. While loss of this tumor suppressor gene is a common event in neoplastic transformation, p21's role in early disease has not been well defined. To that end, we examined the expression of p21 in premalignant mucosa of the AOM-treated rat. We observed that p21 levels were dramatically decreased after 10 weeks post carcinogen injection. At this time, not only was the...
mucosa microscopically normal, but there were no macroscopic lesions elsewhere in the colon (typically adenomas requires 20 weeks and carcinomas 35-40 weeks to develop). The functional significance of an early loss of p21 has been clearly shown in the adenomatous polyposis coli (APC) truncated mouse (analogous to the MIN mouse), in which targeted inactivation of p21 lead to more tumorigenesis (greater number and size) (20). Thus, the early loss of p21 is likely important in carcinogenesis, presumably through impacting the diffuse mucosal hyperplasia. With relevance to early carcinogenesis, p21 could therefore be a natural target for chemoprevention. For instance, loss of p21 (by promoter methylation) in an APC driven mouse model made them resistant to the ability of sulindac (a NSAID) to decrease polypl number and/or size (19). Therefore, there is clear experimental data and biological precedence to support the central role of p21 in antiproliferative and hence chemopreventive effects of PEG.

An unresolved issue of this study is to understand the mechanism of PEG action on p21 as this bulky molecule is unlikely to have direct access to the nuclear compartment. One important regulator of p21 is epidermal growth factor receptor (EGFR). There are several lines of evidence to indicate that EGFR regulates p21 during neoplastic transformation either by phosphorylation and targeting for degradation by the ubiquitin-proteosomal pathway or subjected to transcriptional regulation. This finding is relevant, based on the fact that PEG caused a rapid and sustained internalization of EGFR. Moreover, the effect of PEG on proliferation was suppressed by EGFR inhibition through either RNA interference or pharmacological agent (gefitinib) (13). Thus, our current report in conjunction with previous studies supports a model for PEG's downstream effects as an EGFR -> p21 -> cell cycle arrest - chemoprevention.

Our study is an important first step in understanding how PEG controls proliferation but should not be viewed as an exhaustive study. It is likely that the anti-proliferative effect of PEG may be impacted by the full cadre of G1-S regulators. While our study does not preclude a role for the other modulators, we clearly show that p21 regulation appears to be an important aspect of PEG's downstream effects as a model for PEG -> downstream EGFR effects -> p21 -> cell cycle arrest -> chemoprevention.

In conclusion, we demonstrated that PEG is involved in p21 regulation as a means for its anti-proliferative and hence chemopreventive role in colon carcinogenesis. This and our previous studies lead us to put forth a model where PEG causes internalization of EGFR leading to p21 induction with concomitant G1-S phase cell cycle arrest. Future studies will further elucidate these pathways and determine if occurs in placebo-controlled human trials.

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