Cancer-inducible Transgene Expression by the Grp94 Promoter: Spontaneous Activation in Tumors of Various Origins and Cancer-associated Macrophages

Ramachandra K. Reddy, Louis Dubeau, Heather Kleiner, Tyler Parr, Peter Nichols, Bryce Ko, Dezheong Dong, Howard Ko, Changhui Mao, John DiGiovanni, and Amy S. Lee

Abstract

A major challenge in treating cancer is the difficulty of bringing therapy to poorly perfused areas of solid tumors, which are often most resistant to chemotherapy and radiation. Grp94 is a chaperone protein localized in the endoplasmic reticulum with antiapoptotic properties. We report here that in vitro the proximal murine grp94 promoter is regulated differently from the hypoxia response element fused to the SV40 minimal promoter, with glucose starvation as an inducer of grp94 but a potent repressor of the hypoxia response element/SV40 fusion promoter. Through the use of transgenic mouse models, we showed that LacZ transgene expression driven by the grp94 promoter was strongly activated not only in spontaneous but also in a variety of chemically induced tumors. We additionally discovered that macrophages in the vicinity of malignant tumor showed a high level of transgene expression, consistent with intense β-galactosidase staining at boundaries between viable tumor cells and necrotic areas. Isolated macrophages also showed grp94 mRNA and transgene activation under glucose starvation in vitro. In contrast, transgene activity was not detected in the normal tissue counterparts of any of the malignant tumors examined or macrophages associated with normal organs. These studies provide direct evidence that the tumor microenvironment is a potent physiological inducer of the grp94 promoter. The unique properties of the grp94 promoter suggest that it may offer a novel tool for directing transcription of therapeutic agents to tumors particularly in resistant regions bordering necrotic areas, delivered through standard vectors or macrophages.

Introduction

The mammalian stress response is an evolutionarily conserved mechanism that allows cells to respond to adverse environmental or metabolic conditions. This response is represented at the molecular level by the induced synthesis of specific sets of cellular proteins with protective functions. The GRPs represent one such set of proteins of which the expression is induced by glucose starvation and stress conditions that disrupt ER function or homeostasis (1). GRP94, also referred to as gp96, is a 94 kDa calcium-binding glycoprotein residing in the ER (2). Interestingly, whereas other GRPs are conserved from yeast to human, GRP94 has only been identified in vertebrates (1).

Grp94 is known to function as a chaperone for proteins and immunogenic peptides, and exhibits antiapoptotic properties (2, 3). Overexpression of GRP94 has been associated with cellular transformation (4). In a variety of cancer cell lines, rodent tumor models and human cancer biopsies, the level of GRP94 is elevated, correlating with increased tumorigenicity (5–8). This is consistent with the induction of the GRPs with protective functions as a survival response to nutrient starvation, acidosis, and hypoxia, conditions that are common in poorly vascularized solid tumors (1, 9). Interestingly, it has also been reported that GRPs can be activated in response to altered metabolic states associated with transformed cells independent of the glucose status (4, 10). These unique properties of GRP induction suggest that the grp promoters can be useful for directing therapeutic agents within the tumor microenvironment. Despite supportive evidence in tumor xenograft models performed with cell lines stably transduced with retroviral vectors containing the grp78 promoter (11), the expression profile of any grp promoter in the context of a transgene in adult animals is unknown. Furthermore, the physiological stimuli of such promoters in vivo remain to be determined.

We report here that in tissue culture systems the proximal murine grp94 promoter, which is inducible by glucose starvation, is regulated differently from the HRE (12). To additionally investigate how a transgene driven by the grp94 promoter is regulated in adult animal, we established TG mice containing the mouse grp94 promoter fused to the LacZ reporter gene. We show here that the transgene driven by the grp94 promoter, whereas quiescent in all of the major organs, is strongly activated not only in a wide variety of tumors but also in macrophages in the vicinity of malignant tumors, with intense transgene expression at the border of necrotic areas and viable tumor cells. Isolated macrophages also showed grp94 mRNA and transgene activation under glucose starvation in vitro. These results provide the direct evidence that the tumor microenvironment of a tumor is a potent physiological stimulus of grp94 transcription in adult mammals. Our findings also imply that the grp94 promoter can provide a novel tool for directing transcription of therapeutic agents to solid tumors particularly at chemo- and radiation-resistant regions bordering necrotic areas. Macrophages can form a significant portion of solid tumors and predominate in the regions of tissue necrosis mediating the immune response (13). The activation of the grp94 promoter in cancer-associated macrophages additionally opens up the possibility of “arming” the macrophages to improve its efficiency for cancer therapy (14).

Materials and Methods

Plasmids. The −357/luciferase reporter gene (−357/Luc) was constructed by cloning a PCR-generated murine grp94 promoter subfragment (spanning −357 to +30) into NheloHindIII digested pGL3 basic luciferase vector (Promega Corp., Madison, WI). The MAP11 plasmid containing nucleotides −985

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2 To whom requests for reprints should be addressed, at Department of Biochemistry and Molecular Biology, University of Southern California/Norris Comprehensive Cancer Center, 1441 Eastlake Avenue, Room 5307, University of Southern California Keck School of Medicine, Los Angeles, CA 90089-9176. Phone: (323) 865-0507; Fax: (323) 865-0094; E-mail: amylee@hsc.usc.edu.

3 The abbreviations used are: GRP, glucose regulated protein; β-gal, β-galactosidase; DMBA, 7,12-dimethylbenz(a)anthracene; ERSE, endoplasmic reticulum stress response element; HRE, hypoxia response element; RT-PCR, reverse transcription-PCR; TG, transgenic; ER, endoplasmic reticulum; FBS, fetal bovine serum.
to −939 from the human vascular endothelial growth factor promoter inserted into pGL2-promoter containing the minimal SV40 promoter upstream of the luciferase gene (Promega) was kindly provided by Dr. Gregg L. Semenza (The Johns Hopkins University School of Medicine, Baltimore, MD), and its construction has been described (12).

Transient Transfections. 293T human renal epithelial cells were cultured in DMEM containing 4.5 g/liter glucose and L-glutamine (Mediatech, Inc., Herndon, VA) supplemented with 10% FBS. Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO2. Transfections were performed using Superfect (Qiagen, Inc., Valencia, CA) at 1 µg plasmid DNA/well in six-well plates according to the supplied protocol. As a control for transfection efficiency, cultures were cotransfected with 1 µg of β-gal expression vector (cytomegalovirus β-gal). Each transfection was performed in duplicate, and each experiment was repeated two to three times.

Glucose Starvation and Hypoxia. For glucose starvation, the cells were cultured in glucose-free DMEM (Invitrogen Corp., Carlsbad, CA) supplemented with 10% dialyzed FBS (Life Technologies, Inc., Grand Island, NY) for 24 h. Hypoxic conditions were accomplished by flushing the plating chambers (Modular Incubator Chamber; Billup-Rothenberg, Inc., Del Mar, CA) for 10 min with a mixture of 0.1% O2, 5% CO2, and roughly 95% N2 (Specialty Air Technologies, Inc., Long Beach, CA), sealed, and placed at 37°C. Another chamber containing 20% O2, 5% CO2, and 75% N2 was used for control of the control cells. Cells were harvested 24 h after the hypoxia treatment. Generation of grp94/LacZ TG Mice. A 2.4-kb murine genomic fragment was isolated from a LambdaFix II library derived from the mouse strain 129Sv (gift of Dr. Robert Maxson, University of Southern California Keck School of Medicine) and subcloned into pBlueScript. This fragment contains 1 kb of the grp94 promoter, the first exon encoding the leader sequence (48 bp) and part of the second exon (25 bp). It was used to drive the expression of the LacZ gene derived from the plasmid pNASSβ. A 6-kb Xhol fragment containing the grp94/LacZ transgene was injected into fertilized eggs from superovulated F1 (C57BL/6fCBA/J) females. CD1 pseudopregnant females were used for embryo transfer. Offspring resulting from these zygotes were screened for the transgene by isolating genomic DNA from mouse tail and performing Southern blot analysis.

Chemical Carcinogenesis. The backs of mice were shaved 2 days before treatment and dosed weekly with DMBA (100 nmol/mouse in 0.2 ml acetone). The incidence and multiplicity of both skin papillomas and carcinomas were recorded weekly. Carcinomas were initially recorded grossly as downward invading lesions and later verified histologically. Mice were sacrificed when tumors size was recorded weekly. Carcinomas were initially recorded grossly as downward invading lesions and later verified histologically. Mice were sacrificed when tumors grew larger than 1 cm in diameter or when the animals were moribund. TG and control mice were sacrificed by cervical dislocation.

β-Gal Staining. Tumors or normal tissues were quickly excised, cut into pieces, and fixed in 4% paraformaldehyde (made in PBS; pH 7.5) for 30 min at 4°C. Tissue sections were rinsed three times 10 min each in ice-cold PBS, immersed in freshly prepared staining solution [10 ml solution of PBS (pH 7.4) containing 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% NP40, 0.1% X-gal dissolved in dimethylformamide, 82.5 mg potassium ferricyanide, and 94.5 mg potassium ferrocyanide], and incubated overnight at 37°C. The reaction was stopped by rinsing the organs in PBS. Samples were stored in 70% ethanol at 4°C. β-Gal staining of the macrophages was performed as above with the exception that the cells were fixed in 0.2% gluteraldehyde (made in PBS; pH 7.5) for 20 min and incubated in the staining solution for 48 h.

Macrophage Isolation and RT-PCR. Female B6D2F1/J mice were injected with sterile 3% (w/v) bacitracin/potent i.p. to develop activated macrophages that were isolated by a syringe and plated on sterile glass coverslips. After overnight attachment, separate wells containing the coverslips had medium replaced with fresh DMEM containing 5% FBS and 2 mM glutamine, or in glucose-free DMEM containing 5% dialyzed FBS and 2 mM glutamine. After 16 h, RNA was isolated using TriZol LS (Life Technologies, Inc.) and transcribed into cDNA using Life Technologies, Inc., Superscript II RT-PCR kit. PCR was performed using mouse grp94 and β-actin primers in the linear range of cDNA input. The 471-bp grp94 PCR fragment was primed using 5’ primer TTGAAAAAATGTGGGATGGGG and 3’ primer TGCTCGTATATACTCAGCA. The 220-bp β-actin fragment was primed by a 5’ primer AACACAAAGGATGAGTGTG and 3’ primer AAGGTTCCTACTGGAAGA. PCR was conducted with 35 cycles of a 94°C (30 s), 50°C (30 s), and 72°C (2 min) sequence. Integration of band intensity was performed on a Bio-Rad gel imager using Bio-Rad Quantity One Software.

Results

Differential Response of the grp94 and HRE/SV40 Fusion Promoter to Glucose Starvation and Hypoxia. Overexpression of the GRPs has been reported in solid tumors where nutrient deprivation and hypoxia are common. To dissect the grp94 induction mechanism in vitro, we isolated the murine grp94 promoter, and through DNA sequencing located three conserved activator elements referred to as the ERSE within the first 200 bp proximal to the TATA element. The luciferase reporter gene was fused to the grp94 promoter spanning −357 to +30 (referred below as the proximal promoter). This plasmid, −357/Luc, was transfected into 293T cells. For comparison, the cells were transfected with plasmid MAP11, which contains the HRE of the human VEGF gene fused to the minimal SV40 promoter driving the expression of the luciferase reporter gene (12). We observed that the proximal grp94 promoter is stimulated by glucose starvation, increasing its activity by ~3-fold but not by hypoxia (0.1% oxygen; Fig. 1A). In contrast, the MAP11 promoter activity was severely suppressed by glucose depletion but was enhanced 3.5-fold by hypoxia (0.1% oxygen; Fig. 1A). Thus, in tissue culture cells, glucose starvation is a key inducing agent of the proximal grp94 promoter, whereas hypoxia at 0.1% oxygen is not sufficient for its stimulation.

Quiescent Transgene Expression Driven by the grp94 Promoter in Normal Organs of Adult Animals. In comparing the promoter organization of the murine grp94 promoter with human and chicken sequences, which had been reported previously (15), there is a striking conservation of both the orientation and location of the ERSEs of the three vertebrate grp94 promoters (Fig. 2A). For example, all three of the grp94 promoters contain three ERSEs at near identical locations in relation to the TATA element. In all three of the cases, the two most proximal ERSEs occur in an inverse orientation. Toward understanding in vivo regulation of transgene expression driven by the grp94 promoter, we created grp94/LacZ, which contains 1 kb of the mouse grp94 promoter upstream of the TATA element with a complete array of the ERSEs fused to LacZ as the reporter gene (Fig. 2B). Multiple lines of TG mice with stably integrated grp94/LacZ transgene were established and crossed to homozygosity. Tissues from the grp94 TG mice derived from three independent founder lines and their non-TG siblings were stained for β-gal expression. Organs including the brain, heart, lung, liver, kidney, intestine, spleen, and muscle were stained either as whole organs or sectioned pieces. For all of the animals being examined, we could not detect any specific expression in any of the organs. An example of the β-gal staining pattern for 6-month-old grp94 TG and non-TG mice is shown in Fig. 2C. Although a weak blue stain was detected in the intestine and kidney, a similar pattern of staining was evident in the non-TG siblings or wild-type mice, consistent with bacterial and endogenous β-gal activity present in those respective tissues. Thus, the level of the LacZ transgene expression under the control of 1 kb of the grp94 promoter in adult TG animals was minimal and below the sensitivity of this assay.

Spontaneous Induction of LacZ Transgene Expression in Tumors. As the TG mice grew older, we noticed that some of the mice developed spontaneous tumors. For example, lymphomatous nodules of various sizes were discovered in a 1-year-old grp94 TG mouse. Upon β-gal staining, these tumors showed regions of intense blue staining throughout the tumor (Fig. 2D). To generate a larger number of tumors for examination of transgene expression, grp94 TG mice (from three independent lines) and their non-TG siblings were subjected to a multistage carcinogenesis protocol using DMBA as a chemical carcinogen. After 6 months of DMBA dosing, incidences of skin tumors developed as expected in both
the TG and the non-TG mice. Furthermore, when the mice were sacrificed, various tumors were found in the abdomen and liver. Because the mice that were treated were all 1 year of age, it was possible that some of the tumors formed spontaneously rather than by direct action of DMBA. In any case, this regimen yielded many tumors from TG and non-TG mice.

The normal organs such as brain, spleen, liver, and muscle of both TG and non-TG mice treated with DMBA did not show any β-gal staining.
(Fig. 3A). This, in combination with the result in the younger mice (Fig. 2C), indicates that transgene expression driven by 1 kb of the grp94 promoter is relatively quiescent in the normal organs of both young and old animals. In contrast, the tumors isolated from all three of the TG mouse lines stained intensively for β-gal, whereas β-gal staining of the tumors derived from non-TG siblings was negative or minor (Fig. 3B).

Specific Transgene Expression in Malignant Tumors and Cancer-associated Macrophages. To confirm the neoplastic nature of the tumor sections, histological examinations of the counterstained tumor sections were performed. Diffuse β-gal staining indicative of grp94 promoter activity could be demonstrated in several spontaneous as well as chemically induced malignant tumor sections. An example of β-gal staining of a tumor section derived from a spontaneous malignant lymphoma is shown in Fig. 4A. This section, which shows sheets of monomorphous lymphocytes with morphological features characteristic of a diffuse malignant lymphoma, contains many areas of β-gal staining, some of which are encircled in red. Examples of β-gal staining in neoplastic cells seen at higher magnification are encircled in Fig. 4B.
similar β-gal staining pattern was observed in a chemically induced hepatocellular carcinoma shown in Fig. 4C. In contrast, the normal cellular counterparts of these tumors, including lymph nodes and liver, showed no detectable evidence of transgene expression (Fig. 3A; data not shown). Thus, these findings show an association between the activity of this promoter and the malignant phenotype.

In examining the histological sections, we additionally noted intense β-gal staining at the boundaries between extensive necrotic areas and adjacent viable tissues. This was prominent in two hepatocellular carcinomas associated with extensive areas of tumor necrosis (Figs. 4, D and E). The exact nature of the cells expressing β-gal activity in these sections cannot be accurately determined because of the intensity of the staining and loss of tissue architectural landmarks because of severe necrosis. However, both of these areas contain heavy inflammatory infiltrates, and the distribution of the β-gal staining as well as the absence of viable cancer cells in those areas strongly suggest that the β-gal activity comes from inflammatory cells such as macrophages. This conclusion is supported by the photomicrograph (Fig. 4F), which is taken from a necrotic area of the same tumor (Figs. 4, C and D) showing lesser staining intensity for β-gal and containing better preserved cellular landmarks. Here, β-gal staining can be seen within individual cells (encircled in red in Fig. 4F).

These cells show features most consistent with macrophages such as oval to irregular cytoplasm and numerous cytoplasmic debris suggestive of phagocytosed material.

**Grp94 Induction in Macrophages Subjected to Glucose Starvation in Vitro.** To confirm that macrophages activate grp94 in response to glucose starvation and normally show minimal endogenous β-gal activity, macrophages were isolated by peritoneal lavage. In the first approach, we examined induction of endogenous grp94 mRNA by harvesting macrophages from non-TG mice injected i.p. with peptone. The *in vivo* activated macrophages were either incubated in normal culture medium or glucose-free medium for 16 h. RNA isolated from these cells was subjected to RT-PCR with grp94 and β-actin primers. The inset in Fig. 1B shows increased grp94 transcript in the glucose-starved cells. Normalizing the ratio of grp94:β-actin, *in vivo* activated macrophages cultured in glucose-free medium displayed about a 2-fold increase of grp94 mRNA compared with the same cells maintained in normal medium (Fig. 1B).

Next we examined the issue of endogenous β-gal activity in macrophages and whether the LacZ transgene can be activated *in vitro* through glucose starvation of macrophages. Unactivated (naïve) peritoneal macrophages from both non-TG and heterozygous 94-TG mice were isolated and underwent a 4-day *in vitro* partial maturation period on glass coverslips. After 16 h of glucose starvation or culture in normal medium, the cells were stained for β-gal activity. Minimal or no β-gal stain was observed for both TG and non-TG macrophages cultured in normal medium (Fig. 1C). A low level of β-gal staining was observed occasionally in a small fraction of non-TG cells subjected to glucose starvation, suggesting a low level of endogenous β-gal activity; this was markedly lower or absent in cells plated on plastic rather than glass surfaces (data not shown). Confirming that the LacZ transgene driven by the grp94 promoter can be induced by glucose starvation in macrophages, strong β-gal staining was detected in macrophages isolated from TG mice subjected to glucose starvation (Fig. 1C).

**Discussion**

Despite many reports describing GRP activation by various stress inducers in tissue culture systems and the potential use of the grp78 promoter in suicide gene therapy (1, 11), our knowledge on how a transgene directed by any grp promoter is regulated in an adult animal is not known. Here we generated TG mouse models with LacZ as the reporter gene driven by the grp94 promoter to examine multiple tissues for transgene expression and make several novel observations. Previously, through *in situ* hybridization and immunohistochemical staining of mouse embryos with GRP94-specific probes and antibodies, tissue-specific induction of GRP94 has been reported correlating with rapid cell proliferation and glucose utilization in the developing embryos (16). In organs and tissues of adult animals, we observed the level of grp94 mRNA is much subdued (data not shown). Using the TG mouse model generated in this study, we show here that transgene expression driven by 1 kb of the mouse grp94 promoter is minimal in most major organs and tissues in adult animals. One explanation is that because GRP94 is a stable protein with a long half-life, most cells may only require minimal transcription of grp94 for sustaining normal cell function. Another explanation is that the promoter sequence driving transgene expression may not contain all of the enhancing elements required for high basal level expression. Irrespective of the cause, our studies show that a transgene driven by 1 kb of the murine grp94 promoter is maintained at a minimal level in normal tissues. For gene therapy, this is a major advantage over other viral or cellular promoters that are constitutively expressed at high levels in all tissues.

Whereas expression of the transgene in normal organs in both young and old animals is minimal, we discovered that in spontaneous tumors as well as chemically induced tumors, the induction of the transgene driven by the grp94 promoter is clearly evident. The TG mouse model used in this study has more advantages over previous studies on grp78 where tumors are generated by s.c. injection of cancer cells (11, 17). Because host animals can alter the biology of grafted tumors, spontaneous or induced tumors are more physiologically relevant. Here, we provide direct evidence that the microenvironment of a naturally growing tumor is a potent *in vivo* stimulus for induction of the grp94 promoter. Because the grp promoters are conserved in structure and share common *cis*-regulatory elements (18), results obtained with the grp94 promoter likely apply to other grp promoters.

What are the physiological stimuli that lead to the induction of the grp promoters within malignant tumors? Several explanations are possible. Fast growing tumors that are poorly vascularized are deprived of glucose and often develop hypoxia, which at an oxygen level <0.02% leads to induction of grp78 (9). Furthermore, cancer cells often function anaerobically, resulting in glycolytic generation of acidosis that is known to induce the GRPs (19). Because grp94 is coordinately regulated with grp78 (19), we predict that cancer cells within a solid tumor subjected to these adverse conditions will naturally turn on the grp94 promoter as a cellular defense mechanism. Interestingly, the glucose concentration for grp78 induction in HepG2 cells has been determined to be 1 mM or below, a level that is considerably lower than the basal plasma glucose concentration of 50 mM (20). Thus, grp induction by glucose starvation requires severe depletion of glucose, a condition that in adult animals may occur largely in pathological organs or tissues such as poorly perfused tumors or tumors subjected to antiangiogenesis treatment.

GRP activation can also be part of the response of the cell to altered metabolic states that occur in transformed cells independent of glucose status within the cell. Elevated GRP94 level has been reported in a variety of human cancer cells as well as in cell lines that are transformed chemically or virally (4, 7, 10). Because the cells were maintained in medium containing glucose, induction of the GRPs in these cells cannot be accounted for the lack of glucose. We propose cellular transformation results in altered signal transduction pathways such as the activation of stress kinases leading to grp induction (21). The spontaneous induction of the grp94 promoter in malignant tumor cells, while maintaining quiescence in the normal cellular counterparts, suggests that it can be a novel tool for directing thera-

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peutic gene expression within tumors. Transcription targeting in tumors can be achieved by using tumor-specific or disease-specific promoters. A more general approach is to use cellular promoters that are ubiquitously expressed in a variety of tumors. The latter approach offers the advantage that it can be applied toward the treatment of multiple types of cancers. Despite recent advances in these areas, significant problems remain in achieving efficient delivery of the therapeutic genes to large areas of tumors with deficiencies in tumor blood supply. The problem is compounded because cells in these areas often develop resistance to both chemo- and radiation therapy, and are largely responsible for the recurrence of the tumor after treatment. Our observation that the grp94 promoter is intensely activated in the region bordering necrotic areas and viable tumors suggest that it can be a powerful tool for directing high-level expression of therapeutic gene in these resistant regions.

Another interesting and potentially important observation of this study is that macrophages in the vicinity of malignant tumor showed a high level of transgene expression driven by the grp94 promoter. It is possible that some of the staining was because of uptake of β-gal-containing debris derived from cancer cells. However, the fact that endogenous grp94 mRNA as well as transgene expression driven by the grp94 promoter were induced by glucose starvation in isolated macrophages in vitro strongly suggests that at least some of the staining seen in the vicinity of necrotic areas in vivo was because of grp94 promoter induction by the macrophages themselves. Macrophages can form a significant proportion of solid tumor masses and predominate in the regions of tissue necrosis (13). Although the use of activated macrophages alone in cancer treatment has not been effective, the natural accumulation of macrophages in the necrotic areas as part of the host immune response suggests that they could serve as efficient gene delivery vehicles for therapeutic agents, because macrophages can diffuse to the necrotic sites devoid of vascular supply (13, 14). Whereas future studies are required to determine more precisely the potential immunological involvement in triggering the grp94 promoter, by arming the macrophages with therapeutic genes driven by cancer-inducible promoters such as grp94, expression of therapeutic genes may be directed toward the inflammatory regions of tumors. This will help minimize expression in organs such as liver and lung that also trap for macrophages administered systemically. Furthermore, given our discovery that the proximal grp94 promoter is primarily activated through glucose starvation and/or altered metabolic state of cancer, whereas the HRE is highly responsive to hypoxia, combination of these regulatory elements in a compatible synthetic promoter may achieve optimal potency in the tumor environment where both glucose and oxygen are limiting. These optimized promoters, used in combination with cancer targeting delivery systems, will provide a valuable tool for new therapeutic approaches toward the difficult task of eradication of residual cancer.

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