Melanoma Differentiation Associated Gene-7/Interleukin-24 Promotes Tumor Cell-Specific Apoptosis through Both Secretory and Nonsecretary Pathways

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Abstract

Melanoma differentiation associated gene-7/interleukin-24 (Mda-7/IL-24), a novel member of the IL-10 family of cytokines, uniquely displays cancer-specific apoptosis-inducing activity. Positive results in ongoing phase I/II clinical trials have strengthened the possibility of its utilization as a cancer gene therapeutic. Previous studies document that signaling events leading to Ad.mda-7-induced transformed cell apoptosis are tyrosine kinase-independent. These results suggest that mda-7/IL-24 cancer cell-specific activity could occur through mechanisms independent of binding to its currently recognized cognate receptors and might even occur independent of receptor function. An adenoavirus vector expressing a nonsecreted version of MDA-7/IL-24 protein was generated via deletion of its signal peptide. This nonsecreted protein was as effective as wild-type secreted MDA-7/IL-24 in inducing apoptosis in prostate carcinoma cell lines and displayed transformed cell specificity and localization of MDA-7/IL-24 in the Golgi/endoplasmic reticulum compartments. Our results indicate that mda-7/IL-24-mediated apoptosis can be triggered through a combination of intracellular as well as secretory mechanisms and can occur efficiently in the absence of protein secretion.

Introduction

Melanoma differentiation associated gene-7 (mda-7) was identified by subtraction hybridization in the context of induction of irreversible growth arrest and terminal differentiation of human melanoma cells (1, 2). Transfection of mda-7 into a spectrum of human and rodent tumor cells confirmed potent growth-inhibitory properties, not only in the context of melanoma but also in diverse human cancers (3). In contrast, this antigrowth effect was not apparent in normal cells (3). Structural and sequence homology in addition to functional conservation indicated that this gene belongs to the interleukin (IL)-10 family of cytokines and has therefore been redesignated IL-24 (2, 4–9). Several independent studies have demonstrated that a majority of human cancer-derived cell lines, including melanoma, prostate, breast, cervical, lung, fibrosarcoma, pancreatic, colorectal, and glioblastoma undergo apoptosis when exposed to mda-7/IL-24 (reviewed in Ref. 6). Current studies indicate that the mechanism by which mda-7/IL-24 induces cancer-specific apoptosis-inducing activity is complex, involving multiple signal transduction pathways and intracellular molecules (reviewed in Refs. 6, 10, 11), requiring further clarification.

The mRNA encoding mda-7/IL-24 is ~2 kb and encodes a protein predicted to have a molecular weight of M, 23,800 (2) belonging to the four-helix bundle family of cytokine molecules (8, 9). The open reading frame encodes a molecule that is 206-amino acids in length, which is a precursor form of the ultimate cleaved, post-translationally processed and secreted mature product. There are three consensus asparagine glycosylation residues that are N-glycosylated, resulting in a mature secreted protein showing multiple bands on denaturing protein gel electrophoresis likely because of partial and complete sugar modification on available sites (6, 10). The precursor form of mda-7/IL-24 is cleaved at position 48, the signal peptidase cleavage recognition site, during import into the endoplasmic reticulum (ER) for processing and secretion via passage through the Golgi apparatus and secretory vesicles. The cleaved unprocessed protein has a predicted molecular weight of M, 18,200, and several groups have currently demonstrated processing and secretion of the molecule as predicted by the presence of consensus sites relevant to specific processing events (6, 10).

The present studies were designed to assess the relevance of mda-7/IL-24 secretion in mediating cancer gene therapy relevant biological effects (i.e., cancer-selective cell killing). Our results confirm that signaling events leading to susceptibility to Ad.mda-7- or Ad.SP mda-7-induced apoptosis have a potent intracellular mode of action and that this molecule is active in inducing transformed cell-specific apoptosis even without secretion. Our results also demonstrate significant involvement of extracellular signal-regulated kinase 1/2 (ERK1/2) in mda-7/IL-24-induced transformed cell-specific killing as one of several components potentially contributing to this observed activity.

Materials and Methods

Cell Lines, Adenoviruses, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Viability Assay, Fluorescence-Activated Cell Sorter Analysis, and Cell Counting. All human cancer cell lines were obtained from the American Type Culture Collection (Rockville, MD) other than the human fibrosarcoma 2TGH and its derivatives, which were a kind gift from Dr. George Stark (Cleveland Clinic, Cleveland, OH). The immortalized normal prostate epithelial cell P69 was provided by Dr. J. Ware (Medical College of Virginia, Richmond, VA). Culture and maintenance of cells and construction, propagation, and utilization of adenoviruses were described previously (12). Protocols used for MTT, fluorescence-activated cell sorter, and cell counts were as described previously (12).

Western Blot Analyses. Cell lines were grown on 10-cm plates and protein extracts were prepared with radioimmunoprecipitation assay buffer containing a mixture of protease inhibitors. Fifty μg of protein was applied to 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with polyclonal antibodies to mda-7, phospho-ERK1/2, and total ERK antibodies (12).

Matrigel Invasion Assay. Invasion of C8161 cells in vitro was measured as the capacity of cells to pass through a Matrigel-coated transwell insert (Corning Inc., NY). Briefly, transwell inserts with 8-μm pores were coated...
with Matrigel (1 mg/ml), cells were trypsinized, and 200-µl aliquots of cell suspension (1 × 10⁶ cells/ml) were added in triplicate wells. After 48-h incubation, cells that passed through the filter into the lower wells were quantified by direct counting.

**Bystander Tumor Growth Inhibition Assay.** The lower basal layer of cells (P69) was seeded at 2 × 10⁶/cm² dish and infected at 25 multiplicity of infection with the respective viruses. After 24 h, cells were washed five times with PBS and overlaid with 6 ml of 0.4% Noble agar containing 1 × 10⁸ DU-145 cells. After 14 days of incubation during which overlay cells were refed every 4 days, macroscopic colonies ≥2 mm were scored. Colonies were enumerated from triplicate plates, and values were expressed as an average ±SD.

**Immunofluorescence.** DU-145 and P69 cells were grown in chamber slides (Falcon; BD Biosciences, San Jose, CA) fixed with 2% paraformaldehyde, permeabilized by 0.1% Triton X-100, and then incubated with primary antibodies (antirabbit mdA-7; GM130 (BD Pharmingen, San Diego, CA), lysozyme-associated membrane glycoprotein 1/2 (LAMP1/2; Santa Cruz, CA), calreticulin (BD Pharmingen), and Mitotracker marker (Molecular Probes, Eugene OR). FITC-conjugated donkey antimouse IgG or antirabbit IgG (Molecular Probes) were used for visualization on a Zeiss LSM 510 fluorescence microscope.

**Results**

**Growth Inhibitory Effect of Ad.mda-7 and Ad.SP mda-7 on Prostate Cancer Cell Lines.** Experiments were performed to determine whether infection with an adenovirus vector expressing a non-secreted version of MDA-7/IL-24 protein deleted for the signal peptide (Ad.SP mda-7) could induce growth suppression and apoptosis in prostate tumor cells in a manner analogous to that observed using a full-length mda-7/IL-24 (Ad.mda-7; Refs. 13, 14). Parallel experiments were performed with a normal immortalized untransformed prostate epithelial cell line (P69; Ref. 15) to define potential differential susceptibility to these viruses. These experiments confirmed that infection with both viruses induced comparable killing in susceptible prostate tumor cell lines (PC-3, DU-145, and LNCaP), but as previously reported, using Ad.mda-7 (13) did not affect the viability of P69 cells (Fig. 1A).

Ad.mda-7 or Ad.SP mda-7 infection induced an increase in the proportion of DU-145 cells undergoing apoptosis as reflected by an increase in the proportion of cells with a sub-G₁/G₀ hypodiploid (A₀) DNA content (Fig. 1B), as previously described for Ad.mda-7 (13). Similar results were obtained when LNCaP or PC-3 prostate tumor cells were infected with the two mda-7/IL-24 expressing adenoviruses (data not shown). In contrast, no significant change was observed in the percentage of apoptotic cells after infection of P69 cells with Ad.vec, Ad.SP mda-7, or Ad.mda-7 (Fig. 1B). This data provides further support for equivalent cancer-specific cell killing with Ad.mda-7 or Ad.SP mda-7.

To determine the extent of secretion of MDA-7/IL-24 protein after infection with Ad.SP mda-7 and compare it with wild-type Ad.mda-7, we analyzed the supernatant and pellets of infected cells by Western blotting 24 h after infection (Fig. 1C). Intracellular protein was observed in DU-145 cells in extracts derived from both Ad.SP mda-7 and Ad.mda-7. Secreted MDA-7/IL-24 protein was found in the supernatants only from Ad.mda-7-infected cell lines at 24 h (as well as 48 and 72 h; data not shown). The intracellular fractions of mda-7/IL-24 expressed by Ad.SP mda-7 differed from wild-type Ad.mda-7-expressed protein in that the only band present in both extracts was a lower molecular weight band of ~18,000. The additional higher molecular weight bands seen in the intracellular Ad.mda-7 lane (Fig. 1C) are likely the previously reported post-translationally processed forms of this molecule (16–18). This strengthens the possibility that absence of signal peptide impacts on further post-translationally processing of the mutant protein, including lack of secretion. Because in both cases the killing effect is comparable, it is possible that the active form of protein does not necessarily require processing but might need localization to ER and Golgi compartments of cells to be functional. We have also noted an apparently lower amount of MDA-7/IL-24 protein expression, by Western blotting (Fig. 1C, left panel) with the Ad.SP mda-7 virus compared with Ad.mda-7. The viral titers used in these studies are equivalent, as is the extent of cell killing (Fig. 1, A and B). It is possible that (a) stronger intensity generated by glycosylated protein bands attributable to additional antibody trapping of primary or secondary antibody on sugar residues produces an apparently stronger signal for a given amount of protein compared with unglycosylated molecules (b) lower stability of unglycosylated MDA-7 protein because the sugar modification might contribute to stability results in an overall lower steady-state level of this form of protein. The amount of mda-7/IL-24 mRNA expressed by both viruses is comparable in Northern blot analyses.5 Regardless, the phenotypic effect of these two viruses irrespective of the apparent differences in protein expression level is essentially identical with respect to growth inhibition and apoptosis induction.

We previously demonstrated activation of ERK1/2 in glioblastoma cells upon Ad.mda-7 infection (19). To define whether ERK1/2 activation also plays a role in mda-7/IL-24-induced killing in prostate cancer cell lines we used PD98059, a specific mitogen-activated protein kinase kinase 1 signal pathway inhibitor (19). This pharmacological agent inhibited killing of DU-145 cells to a comparable extent after infection with either Ad.SP mda-7 or Ad.mda-7, whereas a similar experimental protocol did not affect the viability of P69 cells (Fig. 1D). A similar inhibition in cell killing was also apparent in PD98059-treated LNCaP and PC-3 cells infected with both viruses (data not shown). To further substantiate this observation, lysisates of P69 and DU-145 cells, either uninfected or infected with the Ad.SP mda-7 or Ad.mda-7 virus, were analyzed by SDS-PAGE followed by Western blotting with antiphospho-ERK1/2 and anti-ERK (total) antibodies. As shown in Fig. 1D, treatment with Ad.SP mda-7 or Ad.mda-7 promoted ERK1/2 phosphorylation in prostate cancer cell lines, but not in the P69 cell line, correlating cell killing with activation of the ERK1/2 pathway. As documented previously for Ad.mda-7 (12), we observed that the Ad.SP mda-7 virus was capable of inducing apoptosis in cells functionally deficient for Janus-activated kinase/signal transducers and activators of transcription (JAK/STAT) signaling (Ref. 20; data not shown), further indicating functional equivalence.

**Secreted and Nonsecreted Forms of mda-7/IL-24 Inhibit Tumor Cell Invasion.** An additional comparison of the relative potencies of both forms of MDA-7/IL-24 protein focused on their impact on tumor cell invasiveness. For this analysis, the effect of Ad.SP mda-7 and Ad.mda-7 infection on the invasiveness of C8161 cells (metastatic human melanoma cells) was studied. This cell line was chosen because of its well-documented and reproducible invasive capacity in vitro as well as its tumorigenic and metastatic properties in vivo mouse assays (21, 22). Invasiveness was evaluated using a Matrigel-base membrane model that determines the invasion of infected cells through a layer of Matrigel-coated 8.0-µm pore size tissue culture inserts. Infection of C8161 cells with Ad.SP mda-7 or Ad.mda-7 inhibited to a similar extent the ability of these cells to invade through Matrigel-coated membrane inserts as compared with Ad.vec-infected cells (Fig. 2A, top panel). This effect, which was apparent 48 h after infection with the MDA-7/IL-24 expressing adenoviruses, occurred without any apparent effect on C8161 cell...

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growth in monolayer culture (Fig. 2A, bottom panel), thereby confirming that invasiveness was not inhibited because of loss of cell viability. These results show that both constructs inhibit invasion with equivalent potency, providing yet another illustration of similar biological activity of these molecules.

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**Only the Secreted Form of mda-7/IL-24 Displays “Bystander” Antitumor Activity.** Earlier studies in pancreatic cancer cells indicated that MDA-7/IL-24 protein possessed a potent bystander killing activity that exerted growth suppressive and apoptotic effects on nontransduced neighboring tumor cells (23, 24). To determine the extent of bystander...
activity, if any, by the nonsecreted mutant form of the protein versus the secreted form of MDA-7/IL-24, a dual cell culture agar overlay approach was used. For this protocol, P69 cells that are resistant to killing by MDA-7/IL-24, although serving as a source of production of this cytokine, were infected with the different viruses followed by overlaying with agar containing susceptible DU-145 cells (Fig. 2B). Using this strategy, infection of P69 cells with Ad.mda-7 (25 pfu/cell) resulted in a reduction in both the number and size of DU-145 colonies growing in agar. In contrast, infection of P69 cells with Ad.SP mda-7 did not induce a significant alteration in DU-145 anchorage independence nor did it decrease the size of colonies growing in the agar overlay as compared with cells infected with Ad.vec. These studies provide direct support for a role of secreted MDA-7/IL-24 in mediating a “bystander” cancer growth-inhibitory effect.

**Localization of MDA-7/IL-24 to ER/Golgi Compartments.** In view of comparable apoptotic induction obtained with Ad.SP mda-7 versus Ad.mda-7, it was important to determine the location of the signal peptide-deleted MDA-7/IL-24 protein. Therefore, comparative subcellular localization of MDA-7/IL-24 protein was analyzed in DU-145 and P69 cells after infection with the Ad.SP mda-7 and Ad.mda-7 viruses. In these experiments, immunofluorescence detection was standardized at different time points to determine whether postinfection time-dependent changes in localization occurred. We also tried to avoid potentially misleading changes in localization that might occur as a result of the loss of internal membrane integrity because of apoptotic events induced by mda-7/IL-24. Comparison of the immunofluorescence data using different batches of viruses, cells and secondary antibodies performed at independent times, yielded similar reproducible patterns of staining with both viruses; representative data are presented for DU-145 in Fig. 3. Similar localization results were seen with P69 cells (data not shown). MDA-7/IL-24 protein was detected only in extra-nuclear regions of individual cells. Although there was a light background cytoplasmic staining, protein location primarily overlapped that of the ER stained with anticalreticulin (Fig. 3). The colocalization of MDA-7/IL-24 in Golgi apparatus was also detected via colocalization with anti-GM130 staining (Ref. 25; Fig. 3). However, no co-localization of MDA-7/IL-24 in mitochondria labeled with MitoTracker red occurred (Ref. 26; Fig. 3) confirming staining specificity.

**Discussion**

On the basis of a number of factors, including its abilities to selectively induce apoptosis in a large spectrum of human cancer-derived cell lines without harming normal cells (reviewed in Ref. 6), inhibitory effects on the growth of human cancer cell xenografts in nude mice (23, 27), and most importantly its capacity to induce tumor regression after intratumoral injection in human tumors in currently ongoing clinical trials (28, 29), the likelihood of mda-7/IL-24 becoming a mainstream cancer gene therapeutic appears highly probable (6, 30). Consequently, considerable interest now exists in elucidating the mechanism by which mda-7/IL-24 distinguishes between normal and transformed cells. Just how mda-7/IL-24 induces this selective effect is clearly very complex, as underscored by experiments described in this manuscript and elsewhere (12) showing that the molecule can function independently of JAK/STAT signal transduction pathways that are classically involved in cytokine-mediated activities. We have additionally demonstrated by sensitive reverse transcription-PCR methodology that apoptosis can be induced in tumor cells not expressing detectable levels of IL-20/IL-22 receptors that bind to MDA-7/IL-24 (12).

The next logical step in pursuing our initial findings of JAK/STAT independence and potential lack of requirement of receptor binding for the antitransformed cell activity of mda-7/IL-24 (12) was to determine whether the apoptotic effect could be triggered by intracellular fractions (possibly by receptor-independent mechanisms) or if extracellular MDA-7/IL-24 protein (receptor mediated) was mandatory for activity. To achieve this objective, an adenovirus vector was constructed that expresses a nonsecreted version of MDA-7/IL-24 protein by deleting the 48 amino acid signal peptide, and the extent of killing, signal transduction pathway activation, intracellular localization, invasiveness, and bystander growth-inhibitory activity was compared with the full-length mda-7/IL-24. Although most of the analyses

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**Fig. 2. Comparative mechanism of mda-7/IL-24 action after infection of various cell lines with Ad.vec, Ad.mda-7, and Ad.SP mda-7.** A, mda-7/IL-24 inhibits C8161 invasiveness without altering C8161 viability. C8161 cells were infected with 100 pfu/cell of Ad.vec, Ad.mda-7, or Ad.SP mda-7. After 24 h, 1 x 10⁵ cells were allowed to invade for 48 h through transwell inserts (8-μm pores) coated with Matrigel. The cells that invaded through the Matrigel-coated inserts were stained, counted, and photographed under a light microscope at x>20 magnification. Cells viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in parallel to ascer-

**B**, mda-7/IL-24 action after infection of various cell lines with Ad.vec, Ad.mda-7, or Ad.SP mda-7 and overlaid with 1 x 10⁵ DU-145 cells suspended in 0.4% agar. Fourteen days later, with agar medium feeding every 4 days, the number of anchorage-independent DU-145 colonies ≥2 mm was enumerated microscopically. Average number of colonies ± SD from triplicate plates. Qualitatively similar results were obtained in two additional studies. pfs, plaque-forming unit; IL, interleukin.
were performed in human prostate cell lines, additional studies using a spectrum of cancer and normal cell lines, suggest that these observations are equally applicable to other human cancers.

The results presented here provide several independent lines of evidence indicating that the effect of Ad.SP-mda-7 and Ad.mda-7 infection is similar with respect to transformed cell apoptosis induction. In particular, treatment of susceptible prostate cancer cell lines with Ad.mda-7 as well as Ad.SP-mda-7 induces killing to a comparable extent through ERK1/2-dependent and JAK/STAT-independent pathways. The fact that both secreted and nonsecreted forms of MDA-7/IL-24 protein have comparable apoptosis-inducing activity was unanticipated, adding a further level of complexity in understanding how this novel molecule works. Localization of full-length MDA-7/IL-24 protein in the ER/Golgi compartments is consistent with the signal peptide hypothesis (31) and the currently known and predicted secreted cytokine nature of the protein (6, 10). Because the signal-peptideless mutant MDA-7/IL-24 protein does not contain an export signal, it is predicted to remain in the cytosol. We have, however, confirmed through confocal immunofluorescence studies that a significant fraction of this protein is able to enter the ER and Golgi apparatus and that proteins derived from wild-type and mutant virus appear to have overlapping patterns of localization within the cell. It is not possible to rule out cryptic internalization signals that become active in the absence of the actual signal peptide, identity of these cryptic sites being currently unknown. Western blot analyses performed on protein-derived cytosolic and extracellular fractions of cells infected with both viruses indicate that only full-length MDA-7/IL-24 is processed and secreted. It is also possible that adenovirus infection produces relatively large amounts of protein that even in the absence of a specific targeting sequence possesses the ability to cross membranes and accumulate in ER/Golgi because of charge and/or tertiary structure. However, because localization of MDA-7/IL-24 is similar in both normal (P69; data not shown) and cancer (DU-145; Fig. 3) cells, differences in cellular localization of this protein can be excluded as a direct mechanism underlying the differential apoptosis-inducing activity of MDA-7/IL-24 toward cancer cells.

From the mechanistic, apoptosis-induction standpoint, programmed cell death pathways are activated by a diverse array of cell extrinsic and intrinsic signals, most of which are ultimately coupled to an obligatory signal propagation event mediated through mitochondria. In the context of localization of MDA-7/IL-24 to the ER/Golgi, emerging evidence suggests that the ER also regulates apoptosis both by sensitizing mitochondria to a variety of extrinsic and intrinsic death stimuli and by initiating cell death signals of its own (32, 33). The observations presented here, raise the question, based on its apparent propensity for ER localization, whether MDA-7/IL-24 protein induces a recently recognized phenomenon of “ER-stress” that in turn induces proapoptotic events (32, 33). Earlier findings from our group support this hypothesis because induction of the GADD genes is classically associated with the stress response including ER-stress pathways. Induction of GADD genes and further upstream events such as activation of p38 mitogen-activated protein kinase was shown to be induced in a transformed cell-specific manner after Ad.mda-7 infection (34). In addition, we show in the present report that both viruses only in the context of transformed cells also specifically activate the p44/42 mitogen-activated protein kinase pathway. Furthermore, Ad.mda-7 infection produced an up-regulation in inositol 1,4,5-trisphosphate receptor in H1299 cells (35). Inositol 1,4,5-trisphosphate receptor is an intracellular calcium-release channel implicated in apoptosis and localized in the ER. Whereas further investigations to determine the mechanism of specificity of MDA-7/IL-24-triggered ER-stress are clearly needed, this report for the first time identifies the existence of a cellular ER-stress mechanism that can be differentially activated in transformed cells by MDA-7/IL-24 and possibly other agents. This finding uncovers a new intracellular locus that may prove amenable for potential cancer therapeutic targeting.

Taken together, our results indicate that mda-7/IL-24-mediated apoptosis can be triggered through intracellular localization as well as via secretion, and in contexts where both are present, a combinatorial effect is probable. Our results, outlined in a model (Fig. 4), clearly reveal that nonsecreted intracellular MDA-7/IL-24 is also active in inducing transformed cell-specific apoptosis, probably through mechanisms mediated by the signaling pathways transduced through the
APOPTOSIS INDUCTION VIA mda-7/IL-24

Fig. 4. Model illustrating the possible molecular basis of mda-7/IL-24 cancer cell-mediated apoptosis. The effects of known physiological and ectopic overexpression of mda-7/IL-24 are shown on left and right sides of the figure, respectively. Normally, mda-7/IL-24 binds to cognate receptors and activates STAT-1 and -3 transcription factors to mediate pathways affecting cell growth. Because mda-7/IL-24 mRNA and protein are normally seen in subpopulations of immune cells and melanocytes, effects are likely initiated in these cell types but might also affect neighboring nonproducing cells because the protein is secreted. When normally or ectopically overexpressed, current findings in this report indicate localization to the ER/Golgi compartments, whether or not the protein contains a secretory signal. Accumulation of MDA-7/IL-24 protein in this compartment triggers apoptosis that could apparently involve induction of pathways described currently as ER-stress. However, MDA-7/IL-24 additionally acts indirectly on mitochondria to generate reactive oxygen species. A combination of pathways triggered by mda-7/IL-24 results in transformed cell-specific apoptosis. IL, interleukin; ER, endoplasmic reticulum; STAT, signal transducers and activators of transcription; ROS, reactive oxygen species.

ER and Golgi compartments. These newer findings are provocative, although enigmatic, and indicate that much still remains to be learned about the mechanism of action of mda-7/IL-24, both in relation to its cancer-selective killing properties and to its potential immune modulatory functions (6, 30). However, based on the initial successes of this cytokine in phase I/II clinical studies in solid cancers and melanomas (28), this effort is certainly justified and holds promise for developing ways of enhancing the clinical utility of this novel cancer-gene therapeutic in treating diverse human neoplasms (6, 36).

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