Accessory Cells of the Microenvironment Protect Multiple Myeloma from T-Cell Cytotoxicity through Cell Adhesion-Mediated Immune Resistance

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Abstract

Purpose: Cellular immunotherapy frequently fails to induce sustained remissions in patients with multiple myeloma, indicating the ability of multiple myeloma cells to evade cellular immunity. Toward a better understanding and effective therapeutic modulation of multiple myeloma immune evasion mechanisms, we here investigated the role of the tumor microenvironment in rendering multiple myeloma cells resistant to the cytotoxic machinery of T cells.

Experimental Design: Using a compartment-specific, bioluminescence imaging-based assay system, we measured the lysis of luciferase-transduced multiple myeloma cells by CD4⁺ or CD8⁺ CTLs in the presence versus absence of adherent accessory cells of the bone marrow microenvironment. We simultaneously determined the level of CTL activation by measuring the granzyme B release in culture supernatants.

Results: Bone marrow stromal cells from patients with multiple myeloma and healthy individuals, as well as vascular endothelial cells, significantly inhibited the lysis of multiple myeloma cells in a cell–cell contact-dependent manner and without substantial T-cell suppression, thus showing the induction of a cell adhesion-mediated immune resistance (CAM-IR) against CTL lysis. Further analyses revealed that adhesion to accessory cells downregulated Fas and upregulated the caspase-3 inhibitor survivin in multiple myeloma cells. Reconstitution of Fas expression with bortezomib enhanced the CTL-mediated lysis of multiple myeloma cells. Repressing survivin with the small-molecule YM155 synergized with CTLs and abrogated CAM-IR in vitro and in vivo.

Conclusion: These results reveal the cell adhesion-mediated induction of apoptosis resistance as a novel immune escape mechanism and provide a rationale to improve the efficacy of cellular therapies by pharmacologic modulation of CAM-IR. Clin Cancer Res; 19(20); 5591–601. ©2013 AACR.

Introduction

Multiple myeloma (MM) has long been the paradigmatic model for investigating the role of the microenvironment in blood cancers (1). The natural niche of multiple myeloma, the bone marrow, is a milieu of growth factors and cytokines that provides optimal conditions for multiple myeloma cell proliferation and survival. Furthermore, the bone marrow microenvironment contributes to resistance against various therapies. Interaction of multiple myeloma cells with accessory cells of the bone marrow microenvironment induces pleiotropic antiapoptotic mechanisms, thereby rendering multiple myeloma cells resistant to established therapeutic regimens (e.g., glucocorticoids, DNA-damaging chemotherapy; ref. 2), as well as investigational anti-multiple myeloma agents (3, 4). This concept, generally known as cell adhesion-mediated drug resistance (CAM-DR), is considered as one of the major obstacles hindering successful treatment (5–7). Next to these well-documented roles on multiple myeloma cell survival, growth, and drug resistance, recent evidence indicates that the bone marrow microenvironment facilitates multiple myeloma cells to escape the immune system (8). This is particularly important, because despite the therapeutic potential of cellular immunotherapies, such as allogeneic stem cell transplantation or donor lymphocyte infusions (9–11), the majority of patients with multiple myeloma receiving these therapies
translatable biomedical advances in cancer therapy.

Materials and Methods

Cell culture

The luciferase (Luc)-transduced human MM cell lines, U266 and UM9, were maintained in RPMI-1640 (Invitrogen), supplemented with 10% FBS (Integro BV) and antibiotics (penicillin/streptomycin; Life Technologies) as previously described (18). The authenticity was confirmed by partial HLA typing carried out maximal 6 months before the most recent experiment. The BMSC line HS-S and BMSCs from patients with multiple myeloma (pBMSC) or from healthy individuals (hBMSC) were cultured in Dulbecco’s modified Eagle medium (Invitrogen) with 10% FBS and antibiotics. BMSC were used in experiments until passage six. All bone marrow samples were obtained after informed consent and approved by the institutional medical ethical committee. Human umbilical vein derived endothelial cells (HUVEC) were obtained from Lonza and cultured in EGM-2 medium (Lonza).

The HLA-DP4 restricted, minor histocompatibility antigen (mHag)-specific CD4+CTL clone 3AB11; the HLA-A2–restricted mHag-specific CD8+CTL clone HA-1; the HLA-A2–specific CD8+CTL clone 1E4; and the HLA-A2–restricted WT-1–specific T-cell receptor transferred polyclonal CTLs were previously described in detail with respect to their antigen-specific and HLA-restricted cytotoxic capacity against the relevant HLA-matched and antigen-positive multiple myeloma cells (18–21). CTLs were expanded using a feeder cell–cytokine mixture and cryopreserved until use as described (19).

Reagents

RGDw peptide (provided by Prof. P. de Groot; University Medical Center Utrecht, the Netherlands; ref. 22) was used at a concentration of 200 μmol/L. Neutralizing antibody against Fas ligand (BD Pharmingen) was used at a concentration of 10 μg/mL. Bortezomib and YM155 (both Selleck Chemicals) were dissolved in dimethyl sulfoxide at a concentration of 200 μmol/L and 1 mmol/L, respectively, and aliquoted for storage until use. Both were diluted in culture medium to the concentrations indicated in each experiment.

Compartment-specific bioluminescence-based cytotoxicity assays

Accessory cells HS-5, pBMSC, hBMSC, or HUVEC, were plated at a density of 1 × 10^5 cells/well in white opaque flat-bottomed 96-well plates (Costar) in 100 μL culture medium. After an adherence period of 6 hours, luc-transduced multiple myeloma cells were added (1 × 10^4 cells/well).
Where indicated, multiple myeloma cells and accessory cells were co-cultured for 16 to 20 hours before the addition of CTLs. Otherwise multiple myeloma cells and CTLs were added together into the assay. The BLI signal emitted from surviving multiple myeloma cells was determined after 24 to 48 hours using a luminometer (SpectraMax, Molecular Devices) within 20 minutes after the addition of 125 μg/ml beetle luciferin (Promega). The % survival of multiple myeloma cells was calculated using the formula: % survival = (mean BLI signal in the absence of CTLs/mean BLI signal in the presence of CTLs) × 100%. In these assays, multiple myeloma cell survival is a direct reflection of T-cell–mediated lysis and correlates with classical chromium release assays (3).

**Granzyme B ELISA**

The granzyme B (GzB) content of cell-free supernatants was determined using a commercial ELISA kit (Pelipair, Sanquin) according to the manufacturer’s instructions.

**Transwell experiments**

Accessory cells were seeded in 24-well plates at a density of 6 × 10^6 cells/well. After 6 hours, multiple myeloma cells were either added on adherent accessory cells or placed in the Transwell inserts with 0.4 μm pore membrane. After 16 to 20 hours, CTLs were added in the compartments in which the multiple myeloma cells were present. The % survival of multiple myeloma cells was determined after 24 hours as described above. % inhibition of lysis was calculated using the formula: % inhibition = (% survival in presence of accessory cells /% survival in absence of accessory cells) × 100%.

**Western blotting**

To specifically harvest multiple myeloma cells after interaction with accessory cells, we used a previously described reverse Transwell system (23, 24). Briefly, HS-5 cells were seeded at a density of 0.5 × 10^6 cells at the bottom of a cell culture insert with a surface area of 452.4 mm^2, pore size 3.0 μm, and pore density 2 × 10^6 cm^-2 (Greiner Bio-One), which permits cell–cell interaction. After adhesion of the HS-5 cells overnight, the inserts were placed in a 6-well plate upside-down, leaving the adherent HS-5 cells on the lower surface. The multiple myeloma cells were then added at the top layer of the porous surface. After 24 hours, multiple myeloma cells were harvested, checked for HS-5 contamination by fluorescence-activated cell sorting analysis, washed twice with ice-cold PBS, and incubated for 15 minutes at 4°C in lysis buffer (Cell Signaling Technology). Lysates were centrifuged at 14,000 g for 10 minutes at 4°C, and the supernatant was removed and stored at –80°C until use. Protein concentrations were determined by a bicinchoninic acid protein assay (Pierce; Thermo Scientific). Samples were separated by SDS-PAGE and proteins were transferred to Immobilon-FL PVDF membrane (Millipore). After overnight blocking with Odyssey Block Buffer (Westburg), membranes were incubated with primary antibodies for 2 hours at room temperature (anti-survivin and anti-Mcl-1; 1:1000 dilution, Cell Signaling Technology). Blots were visualized by Odyssey infrared imaging (LI-COR Biosciences) using Alexa-labeled antibodies according to the manufacturer’s instructions. Band intensities were quantified using ImageJ software.

**Survivin downregulation by shRNA**

Luciferase-transduced UM9 cells were transduced with lentiviral transduction particles TRCN0000073718, TRCN0000073719, TRCN0000073720, TRCN0000073721, TRCN00000222542 (Sigma-Aldrich) according to manufacturer’s instructions.

**In vivo tumor targeting experiments**

Hybrid scaffolds consisting of three 2- to 3-mm biphasic calcium phosphate particles coated with human mesenchymal stromal cells (MSC) and loaded with Luc⁺ MM cell line UM9 were implanted subcutaneously into RAG2⁻/⁻γc⁻/⁻ mice, as described previously (25). As controls, uncoated scaffolds were implanted in the same mice and multiple myeloma cells were injected directly into the scaffold. Ten days after implantation, tumors growing in the scaffolds were treated with CD4⁺ CTL 3AB11 (5 × 10⁶ CTLs per scaffold), by directly injecting the CTLs into the scaffold. An identical set of scaffolds was left untreated. At the day of T-cell injection, subcutaneous pumps (Alzet 1007D) were implanted which were either filled with PBS or with 2.08 μg/μL YM155 (Selleckchem) diluted in PBS delivering 1 mg/kg/d YM155 continuously. Pumps were removed after 5 days. Bioluminescent imaging was conducted as described previously (25).

**Statistical and synergy analyses**

The differences in triplicate measurements were determined by statistical tests as indicated in the figure legends. P values below 0.05 were considered as significant. Testing the type of interaction (additive, agonistic, or synergistic) between YM155 and CTLs was analyzed using the CompuSyn software program (Combosyn Inc.), which is based upon the Chou–Talalay method (26). The combination index (CI) value was calculated in CompuSyn with the input of CS-BLI measurements of multiple myeloma cell survival for CTL alone, YM155 alone, or the combination in a dose titration of at least 5 doses, titrated around the half maximal effective concentration (EC₅₀) of each individual effector. The calculated CI is a quantitative measure for the degree of treatment interaction, with a CI <1.0 indicating synergy, a CI = 1 indicating additive effects, and a CI >1 indicating antagonism.

**Results**

**Accessory cells inhibit CTL-mediated lysis of multiple myeloma cells**

To evaluate the effect of accessory cells on the T-cell–mediated lysis of multiple myeloma cells, we selected BMSCs and vascular endothelial cells, the two main accessory cell types in the bone marrow microenvironment. We used cloned CD4⁺ and CD8⁺ CTLs that are directed at
difficult to lyse the accessory cells (Supplementary Fig. S1A and S1B). After confirming that these CTLs are unable to lyse the accessory cells (Supplementary Fig. S1A and S1B), we tested them in compartment-specific BLI-based assays for the capacity to inhibit multiple myeloma cell survival through their cytotoxic activity (3), in the absence and presence of adherent accessory cells HS-5, pBMSCs, hBMSCs, or HUVECs (Fig. 1A). As expected, both CTLs mediated effective dose-dependent survival inhibition of the relevant MM cell line. In this experimental setting, the inhibition of survival represents multiple myeloma cell lysis by the CTLs (3), which is also confirmed by our previous findings (19, 21). Coculture with any of the accessory cells significantly inhibited this cytotoxic effect (Fig. 1A).
This inhibitory property of accessory cells was not restricted to mHag-specific CTLs because in similar assays, they also protected the multiple myeloma cells from lysis by the tumor antigen WT-1–specific polyclonal CTLs (Supplementary Fig. S2A).

Accessory cells inhibit CTL-mediated multiple myeloma lysis in a cell–cell contact-dependent fashion

To evaluate the mechanism of the protective effect of accessory cells, we first carried out transwell experiments, in which multiple myeloma cells and accessory cells were either cocultured in direct contact, or separated by micropore membrane inserts. As expected, accessory cells significantly inhibited the multiple myeloma cell lysis by CTLs in cocultures when they were in direct contact with multiple myeloma cells (Fig. 1B). However, when accessory cells were separated by a transwell from multiple myeloma cells, the inhibitory capacity of HUVEC and pBMSC was almost completely lost and that of HS-5 diminished substantially (Fig. 1B). Finally, we carried out extended transwell assays, in which not only accessory cells but also multiple myeloma cells were cultured in the bottom chamber of the transwells (Fig. 1C). Also under these conditions, no inhibition of lysis occurred, indicating that accessory cells protected multiple myeloma cells from CTL-mediated lysis mainly via cell–cell contacts. Therefore, we next examined whether blocking the adhesion of multiple myeloma cells to accessory cells with an RGDw peptide, a soluble ligand for cell surface integrins (27), would abrogate the protection of multiple myeloma cells from CTL-mediated lysis. Preincubation of HS-5 or HUVECs with RGDw peptide, but not with an irrelevant peptide, reduced the adhesion of multiple myeloma cells to HS-5 or HUVEC (Supplementary Fig. S3) and substantially decreased their capacity to protect multiple myeloma cells from CTL-mediated lysis (Fig. 2A and B). These findings emphasized the importance of cell–cell contacts for the capacity of accessory cells to protect multiple myeloma cells from CTLs. However, the inhibitory effect could not be attributed to integrin-dependent cell adhesion alone because mere binding of multiple myeloma cells to fibronectin-, vitronectin-, or laminin-coated wells did not induce an inhibitory effect on CTL-mediated lysis of multiple myeloma cells (Fig. 2C and D).

Accessory cells induce immune resistance as well as immune suppression

CTLs are known to initiate target cell death via granzyme (28) or death receptor-mediated mechanisms (29), both of which require CTL activation. Hence, the accessory cell induced inhibition of CTL-mediated lysis in our assays could be either due to suppression of T cells and/or due to an acquired resistance in multiple myeloma cells against the lysis mechanisms of CTLs. To differentiate between these, we measured GzB excretion and Fas ligand (FasL) expression on CTLs as a marker for T-cell activation upon coculture with multiple myeloma cells and accessory cells. The CD4+ CTL-mediated lysis of UM9 cells was inhibited by the accessory cells (Fig. 3A), without a reduction in GzB release (Fig. 3B) or change in FasL upregulation on the CTLs (Supplementary Fig. S4A). These results excluded T-cell suppression as a mechanism and revealed the development of immune resistance in U266 cells could be either due to suppression of T cells and/or due to acquired resistance in multiple myeloma cells against the lysis mechanisms of CTLs. Development of immune resistance in U266 cells could
not be excluded in these assays. Hence, we addressed this by further analyzing regulators of the apoptotic pathways in both UM9 and U266 upon interaction with accessory cells.

**Fas downregulation as a mechanism of CAM-IR**

To gain insight into the mechanisms of CAM-IR in UM9 cells and to evaluate the possible development of immune resistance in U266 cells, we first investigated the expression of Fas and other death cell receptors such as TRAIL receptors 1 and 2 on multiple myeloma cells in the presence and absence of accessory cells. Incubation with HS-5 substantially downregulated Fas expression in U266, and also, but to a lesser extent, in UM9 cells, but TRAIL receptor expression was not reduced (Fig. 4A). Hence, we evaluated downregulation of Fas expression on multiple myeloma cells by accessory cells as a possible mechanism of immune resistance. The action of bortezomib in this system was probably not restricted to Fas modulation only, because the anti-FasL antibodies did not completely block bortezomib-induced effects on CD4⁺ CTL-mediated lysis of multiple myeloma cells (Fig. 4D).

**Downstream mechanisms of CAM-IR in multiple myeloma cells**

The experiments with bortezomib indicated Fas downregulation as a possible mechanism of immune resistance, but also implicated the existence of other mechanisms. Therefore, we next investigated whether adhesion of UM9 and U266 to accessory cells altered the expression of inhibitors of apoptosis proteins and of Bcl-2 family proteins, all involved in granzyme- and death receptor-mediated apoptotic pathways used by CTLs (31). Consistent with previous reports (32), we observed a modest increase of survivin and Mcl-1 both in U266 and UM9 cells upon coculture with HS-5 (Fig. 5A). To ascertain the role of these proteins in immune resistance, we used the small-molecule YM155, which represses survivin and Mcl-1 levels (ref 33 and supplementary Fig. S6A). Indeed, when we combined YM155 with CTLs in the presence or absence of accessory cells, the CTL-mediated lysis of U266 and UM9 was markedly increased (Fig. 5B) in a synergetic fashion (Supplementary Fig. S6B). To validate the involvement of survivin in CAM-IR, we suppressed the expression of survivin in UM9 cells by lentiviral short hairpin RNA (shRNA) transduction (Fig. 5C). Indeed, survivin shRNA-treated UM9 cells had a substantially lower capacity to induce
CAM-IR (Fig. 5D), confirming the involvement of this antiapoptotic protein in CAM-IR.

**In vivo efficacy of CTL and YM155 combination therapy**

Because the *in vitro* experiments with YM155 were successful in abrogating CAM-IR, we set out to test this combination therapy in a novel *in vivo* humanized mouse model, in which a human bone marrow microenvironment can be generated by subcutaneous inoculation of ceramic scaffolds coated with human BMSCs (25). Thus, we compared the CTL-mediated lysis of U9M cells that were either seeded on humanized scaffolds or on uncoated scaffolds. Consistent with the *in vitro* data, T cells completely inhibited the outgrowth of multiple myeloma cells, which were not in the humanized microenvironment. In contrast, the outgrowth of multiple myeloma cells in the humanized scaffolds was not clearly inhibited by T cells (Fig. 6A), confirming the existence of CAM-IR *in vivo*. We next investigated whether this CAM-IR could be abrogated by combination of CTLs with YM155. Mice were thus treated with CTLs alone, YM155 alone, or both CTLs and YM155. Similar to the first experiment, CTLs were unable to eliminate multiple myeloma cells that were implanted in human MSC-coated scaffolds. YM155 therapy alone did also not show a beneficial effect on tumor growth. In striking contrast, however, the YM155 and CTL combination achieved a substantial...
antitumor effect (Fig. 6B), showing that YM155 can abrogate CAM-IR not only in vitro, but also in vivo.

Discussion

T-cell–based immune therapies represent an important strategy for the treatment of multiple myeloma (9–11). However, their success is often hampered by various immune escape mechanisms. Development of clinically applicable strategies to overcome the immune escape is therefore critical to improve the efficacy of cellular immune therapy (34). Despite the well-established concept that the microenvironment is crucially important in the development of resistance to pharmacologic therapies (3, 35), its contribution to immune resistance, that is, rendering multiple myeloma cells resistant to the cytotoxic effect of CTLs, has received little attention until now. In this study, we focused on this question and showed that interaction of multiple myeloma cells with accessory cells of the bone marrow microenvironment can result in the development of a CAM-IR against CTLs.

We show the existence of CAM-IR mainly in an in vitro coculture system, originally developed to study BMSC-induced drug resistance (3). Monitoring the survival of luc-transduced multiple myeloma cells by CS-BLI offered a number of advantages over flow cytometry- or 51Cr-based cytotoxicity assays: First, it enabled us to selectively monitor the survival of multiple myeloma cells after 24-hour incubation and are depicted for three doses. C, UM9 cells were transduced with shRNA for survivin and survivin protein expression was determined by Western blot analysis. D, untransduced and survivin shRNA-transduced UM9 cells were incubated with and without accessory cells and inhibition of lysis in the presence of accessory cells was assessed. Results are expressed as the mean values of triplicate cultures. Results are representative of three independent assays. Error bars represent the SEM.
induction by BMSCs was in line with our expectations, the involvement of vascular endothelial cells in a similar process was intriguing, because as the main physiologic barrier between tumor cells and circulation, until now vascular endothelial cells have frequently been the subject of other types of studies addressing angiogenesis, migration, and dissemination of multiple myeloma cells (36, 37). Given the high degree of vascularization of medullary and extramedullary multiple myeloma tumors, induction of immune resistance by vascular endothelial cells may represent a hitherto neglected mechanism of immune escape which may require specific modulation toward the development of improved cellular immune therapies. These results also imply that induction of immune resistance is not limited to BMSCs, but may very well be applicable to many different types of accessory cells.

In further analyses, we observed that similar to CAM-DR (38), cell adhesion was an essential and major contributor to the induction of CAM-IR: the immune resistance could be significantly suppressed by inhibiting the adhesive interactions between accessory and multiple myeloma cells with the RGDW peptide, a known blocker of multiple integrins. However, unlike CAM-DR (39), CAM-IR could not be induced by sole binding of multiple myeloma cells to fibronectin, vitronectin, or laminin. Signals initiating CAM-IR are therefore most likely triggered by other receptor-ligand systems than the ones tested here, and require further investigation.

To differentiate immune resistance from immune suppression, we measured granzyme B excretion from and upregulation of FasL on CTLs as T-cell activation markers. Although accessory cells did not influence the activation of CD4+ CTLs in both assays, the granzyme B secretion by the CD8+ CTLs is diminished, indicating suppressed T-cell activation. However, the Fas upregulation was not influenced, indicating a partial or may be a split suppression. More importantly however, even in the presence of this partial T-cell suppressive environment, we could show that CAM-IR can be effectively abrogated with the use of survivin/Mcl-1 inhibitor YM155 (Fig. 5B and Supplementary Fig. S6C). In the light of these results, we postulate that modulation of CAM-IR by itself, even in an immune suppressive microenvironment, may be sufficient to improve the efficacy of cellular immune therapies.

Figure 6. In vivo antitumor effect of YM155 and CTL combination therapy. In (A) hybrid scaffolds coated with human MSCs and loaded with LUC+ MM cell line UM9, or uncoated scaffolds, which were in vivo loaded with tumor cells, were implanted subcutaneously into RAG2−/−γc−/− mice. Ten days after implantation, the scaffolds were injected with 5 × 10⁶ CD4+ CTLs or left untreated. In (B) groups of mice (n = 3) were implanted with human MSC-coated and luc+ MM cell-loaded scaffolds. On day 10, 5 × 10⁶ CD4+ CTLs were injected directly into the scaffolds (“T cells” and “T cells + YM155 combination” groups), and/or a subcutaneous pump delivering YM155 at a rate 1 mg/kg/d for 5 days (“YM155” and “T cells + YM155” groups) and/or a subcutaneous pump filled with PBS (“no treatment” and “T cells” groups). Tumor load of each individual scaffold was assessed by BLI. Results are expressed as the mean tumor load in each scaffold. The error bars represent the SEM.
Although the full spectrum of signaling pathways initiating and establishing CAM-IR remains to be characterized by ongoing investigation, our results already indicate that CAM-IR can develop through alterations in the activity of multiple levels of the apoptosis signaling pathways. Downregulation of Fas seems to be one of the mechanisms for CAM-IR. We have shown that this mechanism can be modulated by bortezomib, which significantly augmented the CTL-mediated lysis of multiple myeloma cells even in the presence of accessory cells. Nonetheless, it is notable that bortezomib may modulate multiple, cell-adhesion–mediated or even cell-adhesion–independent mechanisms of immune resistance, as it augmented the CTL-mediated lysis also in the absence of accessory cells. Furthermore, these effects were not completely abrogated by FasL antibodies in all CTL–multiple myeloma combinations we have tested. Indeed in our further exploration, we discovered another, more downstream mechanism of CAM-IR, which could be effectively modulated with the use of the small-molecule inhibitor survivin, shRNA-mediated downregulation of survivin at the mitochondrial level (40, 41). We did not investigate the role of Mcl-1 in CAM-IR, but focused on the attribution of survivin. shRNA-mediated downregulation of survivin in U269 cells attenuated accessory cell-induced resistance.

In conclusion we showed, to our knowledge, the first in vitro and in vivo evidence suggesting that T-cell–based immune therapy can be hampered by the multiple myeloma cell interaction with nonmalignant cells of the local microenvironment of multiple myeloma, not only because of immune suppressive mechanisms, but also through a cell–cell contact-mediated immune resistance mechanism. These observations indicate that future preclinical studies on the impact of T-cell immunity against tumor cells should be using assays, in vitro and in vivo, which incorporate nonmalignant accessory cells of the tumor microenvironment. We also show the feasibility of modulating CAM-IR through a pharmacologic agent. This provides a rationale for further studies to evaluate the feasibility, safety, and efficacy of such CAM-IR modulating agents in combination with immune therapies.

Disclosure of Potential Conflicts of Interest
M.C. Minnema has honoraria from speakers’ bureau from Jansen Cilag. No potential conflicts of interest were disclosed by the other authors.

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