Video Article

Intra-iliac Artery Injection for Efficient and Selective Modeling of Microscopic Bone Metastasis

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Abstract

Intra-iliac artery (IIA) injection is an efficient approach to introduce metastatic lesions of various cancer cells in animals. Compared to the widely used intra-cardiac and intra-tibial injections, IIA injection brings several advantages. First, it can deliver a large quantity of cancer cells specifically to hind limb bones, thereby providing spatiotemporally synchronized early-stage colonization events and allowing robust quantification and swift detection of disseminated tumor cells. Second, it injects cancer cells into the circulation without damaging the local tissues, thereby avoiding inflammatory and wound-healing processes that confound the bone colonization process. Third, IIA injection causes very little metastatic growth in non-bone organs, thereby preventing animals from succumbing to other vital metastases, and allowing continuous monitoring of indolent bone lesions. These advantages are especially useful for the inspection of progression from single cancer cells to multi-cell micrometastases, which has largely been elusive in the past. When combined with cutting-edge approaches of biological imaging and bone histology, IIA injection can be applied to various research purposes related to bone metastases.

Video Link

The video component of this article can be found at https://www.jove.com/video/53982/

Introduction

Metastases account for over 90% of deaths caused by solid tumors. Bone is the most common organ affected by metastases of various cancer types, especially breast and prostate cancers. When diagnosed in the clinic, bone metastases usually have already entered advanced stages with either osteolytic or osteoblastic alterations in bone, often accompanied with neurological symptoms.

Previous studies predominantly focused on the overt osteolytic bone metastases[1-3], however we currently have limited understanding of micrometastases in bones before the onset of the osteolytic process. This is at least partly due to lack of appropriate experimental models and approaches. Genetically engineered mouse models of breast cancer often metastasize to lungs, but much less efficiently to bones[4]. Likewise, the orthotopically transplanted tumors rarely develop spontaneous bone metastases, with some bone-tropical 4T1 mammary carcinoma sub-clones and MSP overexpressed *PyMT* transgenic mouse model as exceptions[5-7]. Intra-tibial drilling can deliver cancer cells to the bone[8-10], but it also incurs damage and inflammation to local tissues. Currently intra-cardiac injection of breast cancer cell lines has been the major approach to investigate bone colonization[11-13]. However, after cancer cells are introduced into left ventricle only a limited proportion will finally reach bone and bone marrow, making it difficult to track microscopic metastases in a quantifiable fashion.

In this study, we establish a technique, namely intra-iliac artery (IIA) injection[14], to selectively deliver cancer cells into hind limb tissues, thereby enriching cancer cells in bone and bone marrow without causing damage to local tissues. Because of the bone specificity, this approach also allows enough time for indolent cancer cells to eventually colonize before the animals succumb to primary tumors or metastases in other vital organs. When combined with a variety of other techniques, such as bioluminescence imaging, immunofluorescence staining and bone histomorphometry, IIA injection is potentially useful for a wide scope of research purposes related to bone metastases, especially to track the progression from single cancer cells to multi-cell micrometastases. In particular, we demonstrated that IIA injection enables us to visualize the interactions between cancer cells and various types of surrounding cells in the bone microenvironment.

Protocol

All animal work was done in accordance to the animal care guidelines of the Baylor College of Medicine.

1. Cell Preparation

Note: Different cancer cell lines can be used for IIA injection depending on research purposes. We have used breast cancer cell lines MCF7, 4T1, 4T07, MDA-MB-361, MDA-MB-231, MDA-MB-436 and prostate cancer cell line C4-2 in our research. We typically use both GFP- and firefly luciferase-labeled cancer cells for our study and show some data here from the GFP-Luciferase-labeled MCF7 cell line.

1. Maintain cells in DMEM containing 10% FBS and 0.1 mg/ml penicillin-streptomycin at 37 °C in 5% CO_2.
2. Before IIA injection, trypsinize cells at 80 - 90% confluence with 0.25% Trypsin/EDTA solution. Use cold PBS to wash cells twice, and re-suspend cells in 10 ml PBS for cell counting. To remove PBS, centrifuge cells at 800 x g for 5 min.
3. Re-suspend cells at a concentration of 5 x 10^6 cells/ml in ice-cold PBS.
4. Use 100 µl GFP- and firefly luciferase-labeled cancer cells for IIA injection of one mouse.
   Note: Therefore, the number of cells received by each animal is 5 x 10^5. This number may need to be altered based upon the aggressiveness of cell lines.
5. Keep cell suspensions on ice.
   Note: Vibrations may be needed to prevent cell aggregation every few minutes until it is ready for injection. For some cell lines (such as MDA-MB-231), more stringent procedures (*e.g.*, passing through 45 µm cell strainer) may be needed to prevent aggregation. Please note that the clog of vessels may cause tissue necrosis, and therefore confounding the experiments.

2. Animal Preparation

1. For animal pain management, give 5 mg/kg/day carprofen (or other analgesic) with dietary supplement to the mice no less than 24 hr before surgery. Administer a dose of 0.1 mg/ml buprenorphine subcutaneously 60 min prior to the surgery. Note: an optional procedure is to implant estradiol pallet into the back of animals to provide extra estradiol. This will accelerate the grow of ER+ cancer cells (*e.g.*, MCF-7 cells)[9].
2. Anesthetize a 4 - 6 week old mouse (approximately 20 g) with ketamine (80 - 100 mg/kg) and xylazine (10 -12.5 mg/ml) by intraperitoneal injection.
3. Confirm the appropriate level of sedation of a 20 g mouse by toe pinch and the observation of a 50% reduction in respiratory rate and pink mucous membranes (*e.g.,* mouse ear skin and paw skin). Keep monitoring these vital signs every 15 min during the surgery. Note: No movement of the animal indicates that the animal is sufficiently anesthetized and ready for surgery.
4. Use vet ointment on eyes to prevent dryness.
5. Shave the mouse on the lower abdomen, especially the right groin area where the surgery and injection will take place.
   Note: This is not necessary if athymic nude mice are used.
6. Put the mouse on its back, spread the legs in their natural positions and stick the toes to mobile dissection cardboard with tape.
7. Wipe the right groin area with 70% isopropyl ethanol soaked sterile cotton swabs, followed with betadine surgical scrub several times.

3. The Common Iliac Vein and Artery Location and Separation

1. Make a skin incision of 1.0 - 1.2 cm long between the 4^{th} and 5^{th} nipples in the lower right abdomen using sterile, #10 carbon steel scalpel blades held in a No. 3 handle. Then use a sterile surgical drape to cover the animal body except the incision site.
2. Move the animal to standard bench top dissection microscope. Use a magnification of 4X for injection.
3. Under 4X magnification of the dissection microscope, insert blunt separation forceps between the fatty tissue and the peritoneum, and push the tissues outwards on both sides to expose the iliac vessels and nerves (see **Figure 1**).
   Note: The artery between aorta from the midline and the lower part of artery branches is called common iliac artery. This is where the injection takes place.
4. Use one pair of straight fine forceps to break through the connective tissue (like a membrane) between vessels and the nerve, closer to the vessels.
5. Switch the straight fine forceps to the left hand. Grab a pair of angled fine forceps using the right hand. Insert the right hand forceps into same position where connective tissues have been broken. And then stick the right hand forceps through, beneath the vessels.
6. At the same time, insert the left hand forceps into the connective tissues on the left side of the vessels to guide the right hand forceps going through.
7. Once the right hand forceps gets beneath the vessels, try to separate the vessels from the surrounding tissue a little bit more, and then keep them on top of the right hand forceps.
8. Use left hand forceps to deliver the tip of a 4-0 silk suture to the right hand forceps, and then pull the suture gently and slowly through beneath the vessels. Note: Now both common vein and artery vessels are lifted up by the suture (see **Figure 2**).

4. Injection and Post-injection Care

1. Prepare the cells for injection in a 31 G insulin syringe. Use 100 µl of cell suspension in PBS per injection. Make sure to pipette up and down several times to separate the cells and avoid aggregation so that the injected cells will not clog and block the blood flow.
2. With the angled forceps in the left hand, put the forceps beneath the vessels along the guidance of the suture, and then open the two arms of the forceps, having the vessels held gently on the forceps.
   Note: The interval between the two arms of the forceps will be the injection site.
3. Hold the 31 G needle with 100 µl cell suspensions in the right hand, with the bevel of the needle upward, ready for injection.

4. Insert the needle into the artery lumen (blood will enter the needle tip). Then push cell suspension slowly in to inject the cells. The cell suspension will push away the red blood in the vessel. Try not to break the vessels or leak the cell suspension out.
5. When the injection is finished, gently pull back the needle and keep holding the vessels up on the left hand forceps to stop bleeding. Then pull away the suture.
6. Pull away the left hand forceps, and quickly use a cotton swab to press the artery incision area to stop bleeding.
   Note: Usually 5 - 10 min continuous pressure is sufficient to stop bleeding.
7. When bleeding stops, use skin glue to close the skin edges of the surgery area. Make an ear-tag, and check for bioluminescence signaling to examine the distribution of injected cells.
   Note: A successful injection will result in an image similar to **Figure 3**.
   1. Check bioluminescence signaling by injection of 100 µl 15 mg/ml luciferin in PBS at 75 mg/kg mouse concentration via the intra-orbital sinus.
      1. For intra-orbital injection, put fingers on both sides of the mouse eye, use pressure to make eye ball slightly pop out from the eye frame, insert 28 G insulin syringe needles between eye ball and nose, and then slowly push syringe to inject luciferin.
         Note: At the correct position, the needle should be able to reach a depth of 2 - 3 mm before hitting hard tissue (bone).
      2. Place the mouse into imaging system for *in vivo* whole animal imaging for 10 - 60 sec within 5 min (see **Figure 3**).
         Note: An optional procedure is to implant estradiol pellet into the back of animals to provide extra estradiol. This will accelerate the growth of ER+ cancer cells (*e.g.*, MCF-7 cells)[15].

8. Leave the mouse on a warm heating pad until it wakes up. Monitor the bleeding, swelling, signs of dehiscence and pain during the post-surgical period. Put the mice back to the cage with analgesic coverage (carprofin dietary supplement suggested) given for pain management until no signs of pain. Pay close attention and care to the animals during 7 days following surgery.

5. Monitoring Metastatic Growth

1. Histomorphometry:
   1. Harvest tumor bearing bone tissues, fix the bones in 4% paraformaldehyde for 24 hr, then decalcify them in pH 7.0 14% EDTA solution for 3 - 5 days, and subject them to paraffin embedding as previously described[14].
   2. Section the paraffin-embed bone tissues at the thickness of 3 µm and perform standard histological method of Hematoxylin/Eosin staining as previously described[14].

2. Immunohistochemistry:
   1. Subject paraffin-embedded tumor bearing bone slides to immunohistochemistry staining for various purposes as previously described[14]. Briefly, immunostain MCF7 cancer cells by anti-GFP antibodies, and immunostain osteogenic cells (pre-osteoblasts and osteoblasts) by anti-Osterix antibodies and anti-Alkaline phosphatase (ALP) antibodies, respectively.

Representative Results

Figure 1 illustrates the anatomical location and relationship of common iliac artery (red) and vein (blue).

Figure 2 shows relative position of iliac vessels and nerves under dissection microscopy. As depicted in **Figure 2A**, the vessels and nerves are right beneath the peritoneal wall and can be revealed after the skin incision is made and the peritoneum is pushed away. The common iliac vein is on the left, and is bigger and darker compared to the artery. The artery is in the middle, and looks pink. It is thinner than the vein but has thicker muscle wall. The vein and artery are parallel and closely connected with each other. Farther on the right is the white lumbosacral nerve. **Figure 2B** shows common iliac vessels separated from the surrounding connective tissue, muscles and nerves, and lifted up by a 4-0 silk suture. The common iliac vein, artery, and lumbosacral nerve are also indicated.

Figure 3 shows representative *in vivo* and *ex vivo* bioluminescence images of animals after intra-iliac artery injection. Five x 10^5 GFP-luciferin-labeled MCF7 cells in 100 µl were administrated to the right hind limb of the mouse by intra-iliac artery injection. D-luciferin was then administrated by intra-orbit sinus injection, followed by the *in vivo* whole animal bioluminescence imaging. The injected MCF7 cells were enriched at the right hind limb of the mouse as indicated by the bioluminescence signals (**Figure 3A**). The *in vivo* bioluminescence signals of the whole animal were tracked every 3 days or every week, and then the mouse bone tissues were harvested at day 14 post-injection when the whole animal bioluminescence signal reached a certain threshold (Photon flux >10^4). After D-luciferin administration, the bone tissues from intra-iliac artery injected mouse were quickly harvested and immersed in PBS for *ex vivo* imaging. The strong bioluminescence signal from the intra-iliac artery injected right hind bone but not from the left control bone showed the specific localization of injected cells (**Figure 3B**).

Figure 4 shows representative images of histological and immunofluorescent staining. When the tumor bearing bones were harvested, they were subjected to paraformaldehyde fixation, EDTA decalcification, and then paraffin-embedding. Three µm bone slides were prepared and standard H&E staining were performed. The compact cobblestone-like cells with larger nuclei were microscopic MCF7 metastatic lesions in the bone tissue 14 days after intra-iliac artery injection, as indicated by the red arrows. Bone marrow (BM) and the large pink flat trabecular bones (TB) with sparse nuclei are so labeled (**Figure 4A**). **Figure 4B** shows GFP-labeled MCF7 cells (green), ALP-labeled osteoblasts (red in the left image), and Osterix-labeled pre-osteoblasts (red in the right image) after immunofluorescent staining. Blue DAPI staining indicates the nucleus.

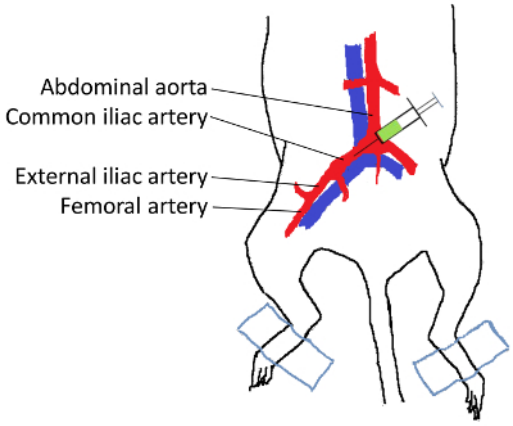

Figure 1: Anatomy of common iliac artery (red) and vein (blue) in mouse. Abdominal aorta, common iliac artery, external iliac artery, and femoral artery are shown as indicated. Injection is performed at common iliac artery from aorta toward femoral artery direction. Please click here to view a larger version of this figure.

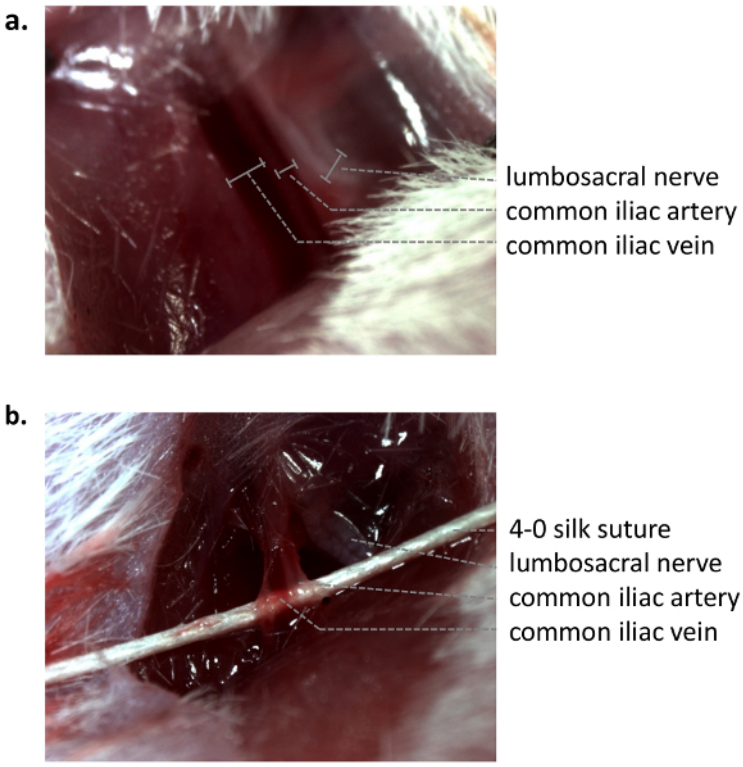

Figure 2: Iliac vessels and nerves under standard dissection microscope with 4X magnification. (**a**) Image of the intact vessels and nerves. (**b**) Image of lifted vessels. Please click here to view a larger version of this figure.

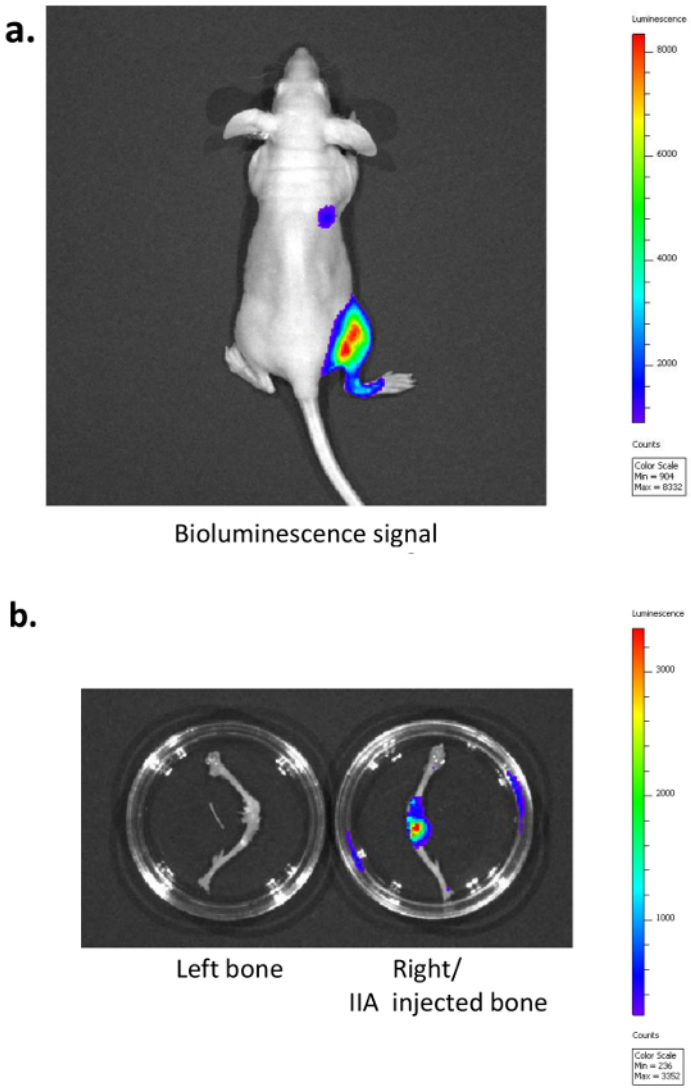

Figure 3: Representative *in vivo* and *ex vivo* bioluminescence images of mouse after intra-iliac artery injection. (**a**) The *in vivo* bioluminescence signal of the whole animal right after injection. (**b**) The *ex vivo* bioluminescence signal of left control bone and the right injected bone from injected animals that were harvested 14 days later. All the bioluminescence signals were measured by following the manufacturer's recommended procedure and settings. Please click here to view a larger version of this figure.

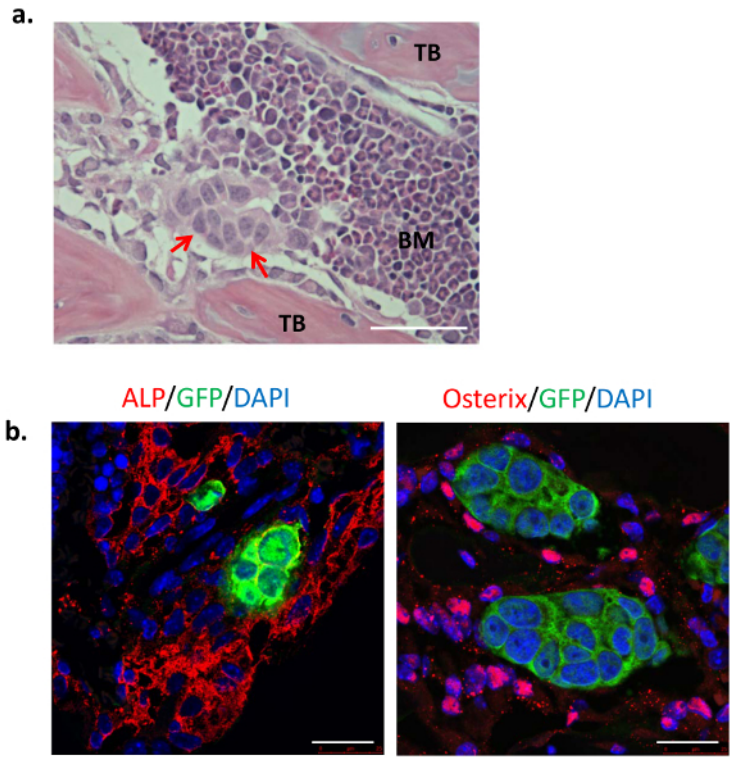

Figure 4: Representative images of histological and immunofluorescent staining. (**a**) Representative H&E staining image of MCF7 tumor bearing bone tissue after intra-iliac artery injection. Scale bar = 25 μm. Note: HE staining is not the effective way to detect tumors. In lower magnification, HE staining is not sensitive enough to distinguish tumors. In higher magnification, it is not efficient to scan all the areas. (**b**) Immunofluorescent staining images of the MCF7 tumor bearing bone tissue. Green: GFP-labeled MCF7 cells, Red in the left image: ALP-labeled osteoblasts, Red in the right image: Osterix-labeled pre-osteoblasts. Blue DAPI staining indicates the nucleus. Scale bar = 25 μm. Please click here to view a larger version of this figure.

Discussion

Although only the iliac artery is the target of injection for cancer cells, we recommend the separation of both iliac vein and artery from surrounding tissues, and to lift them together as a bundle. This is because the vein and artery extensively contact with each other, and the venous vessel wall is thin and is easy to break. Therefore, for a successful injection, it saves time and effort to hold up the two vessels together, although cancer cells are injected only to the artery. A 4-0 silk suture is used to help this process as shown in **Figure 2**. The suture may also help stop bleeding should it occur.

Most steps of IIA injection need to be performed under the dissection microscope. The mouse vessels are soft and small, which makes the procedures challenging. However, after sufficient practice, the success rate can reach 90 - 100% in our experience.

With this technique, researchers may be able to establish bone colonization models of their favorite cancer cell models, including those traditionally thought "non-metastatic". MCF-7 cells represent such an example. Indeed, when arriving in the bone microenvironment, MCF-7 cells undergo a short dormancy before taking off to colonize. Very few spontaneous bone metastases have been detected in animals carrying orthotopic MCF-7 xenografts. However, failure may well result from the residual quantity of disseminated tumor cells, the slow initiation of colonization, and the more aggressive growth of orthotopic tumors (that kills animals before bone lesions can establish). Thus, lack of detection cannot be taken as evidence against MCF-7 cells' ability to metastasize. In fact, indolent or even dormant bone micrometastases may be more prevalent in human breast cancer patients, as suggested by years-to-decades dormancy that is often seen in the clinic.

IIA injection can be applied not only to luminal or basal breast cancer cells, but also to other cancer types such as prostate cancer. When combined with different choices of techniques monitoring bone disease, intra-iliac artery injection can make a significant contribution to many research purposes. We commonly use bioluminescence signaling to trace bone metastasis progression after intra-iliac artery injection, and then harvest tumor-bearing bones for fluorescent immunohistochemistry staining to define interactions between cancer cells and surrounding bone microenvironment niches. Bone histomorphometry[16-17] and μCT[18-20] can be used to evaluate bone structure alterations and other anatomical details of bone that are caused by the inoculation of cancer cells. Proper considerations and combinations should be determined by each group for their specific research purpose.

Similar to intra-tibial[8-10] and intra-cardiac inoculation[11-13], a caveat of intra-iliac artery injection is that it does not recapitulate early steps in metastatic process prior to embolism and entry of tumor cells into the circulation. Ideally, a spontaneous metastasis process starting from orthotopic tumors would be needed to fully recapitulate the metastasis cascade. However, this process is highly inefficient in most models, with only a few exceptions such as some bone-tropic 4T1 sub-clones[5-6] and MSP overexpressed PyMT transgenic mouse model[7]. We hope the IIA

injection will provide novel insights into the bone colonization process, which can in turn facilitate the design and development of truly efficient spontaneous bone metastasis models for pre-clinical studies.

Disclosures

The authors have nothing to disclose.

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