Video Article

A Model of Disturbed Flow-Induced Atherosclerosis in Mouse Carotid Artery by Partial Ligation and a Simple Method of RNA Isolation from Carotid Endothelium

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Abstract

Despite the well-known close association, direct evidence linking disturbed flow to atherogenesis has been lacking. We have recently used a modified version of carotid partial ligation methods [1,2] to show that it acutely induces low and oscillatory flow conditions, two key characteristics of disturbed flow, in the mouse common carotid artery. Using this model, we have provided direct evidence that disturbed flow indeed leads to rapid and robust atherosclerosis development in Apolipoprotein E knockout mouse [3]. We also developed a method of endothelial RNA preparation with high purity from the mouse carotid intima [3]. Using this mouse model and method, we found that partial ligation causes endothelial dysfunction in a week, followed by robust and rapid atheroma formation in two weeks in a hyperlipidemic mouse model along with features of complex lesion formation such as intraplaque neovascularization by four weeks. This rapid *in vivo* model and the endothelial RNA preparation method could be used to determine molecular mechanisms underlying flow-dependent regulation of vascular biology and diseases. Also, it could be used to test various therapeutic interventions targeting endothelial dysfunction and atherosclerosis in considerably reduced study duration.

Video Link

The video component of this article can be found at https://www.jove.com/video/1861/

Protocol

1. Partial ligation of the left carotid artery

1. Mice are used at ~ 8 weeks of age. The weight of the mice is recommended to be at least 18 grams.
2. Sterilize the instruments for 30 second in bead instrument sterilizer and allow to cool down
3. Induce anesthesia with Ketamine (80mg/kg) and Xylazine (10mg/kg) intraperitoneal (i.p.) injection or alternative anesthesia as approved by IACUC. For i.p. injection, inject mouse in the left low quadrant of abdomen.
4. Place mouse in a warming chamber until completely anesthetized. Verify depth of anesthesia with response to toe pinch.
5. Place mouse on a surgical stage, in supine (belly side up) position. Tape the fore-paws palms up and the hind-paws sole down to the stage.
6. Apply liberal amount of depilatory agent (e.g. Nair) to neck between the mandible and the sternum. Gently massage until all hair is removed.
7. Clean off the depilatory agent and apply liberal amount of Betadine to depilated area.
8. Prepare 1 inch-long sections of 6-0 silk suture for ligations. Each mouse will need two pieces of suture.
9. Using a sharp small scissors, make a 4-6mm vertical incision in the middle of the neck. Both the skin and the underlying fascia need to be cut.
10. Move the left parotid gland from the midline to the left and start bluntly dissecting just right of the trachea. You should be able to identify the pulsating left common carotid easily.
11. Follow the common carotid caudally until you locate the bifurcation.
12. Bluntly dissect the bifurcation to expose the following four distal branches off the left common carotid artery: external carotid artery, internal carotid artery, occipital artery and superior thyroid artery. These vessels, especially superior thyroid, can be fragile. Be very careful with your dissection.
13. Tie off the external carotid ABOVE the superior thyroid using 6-0 silk suture. To do this, pass one forceps underneath the artery and grab a piece of precut suture with the help of your other forceps. Gently pull the suture to pass underneath the artery. Tie firmly avoiding any surrounding tissue being caught in the knot. If you tie below the take off of the superior thyroid artery, by the end of the procedure you will effectively have performed a complete ligation which is a substantially different model.
14. Tie off the internal carotid and occipital arteries with one knot using the same technique.

15. Verify position of sutures and patency of superior thyroid artery.
16. Approximate the skin and close skin using a small amount of Tissue-Mend.
17. Place mice in a warming chamber until recovery. Recovery typically takes 30 to 60 minutes.
18. A single dose of Buprenorphine (0.1mg/kg) should be administered immediately post surgery if the anesthesia used does not provide prolonged pain relief (ie. inhaled isoflurane) or if animal is in distress within the first 24 hours. In our experience, the Ketamine dose administered for anesthesia provides sufficient analgesia when surgery is performed by experienced surgeon and no further analgesia is needed.

2. Carotid ultrasound examination

1. To validate whether the partial ligation induced disturbed flow, carotid flow should be examined one day post-ligation
2. Induce anesthesia with isoflurane - anesthesia induction box could be used if needed
3. Place anesthetized mice on imaging stage, belly side up
4. Tape arms and legs to ECG sensor on imaging stage
5. Defoliate neck by liberal application of Nair (this step is only needed if not already done for ligation procedure)
6. Insert rectal thermometer to record temperature
7. Apply Echo gel to neck
8. Start in B-mode.
9. Imaging plane should be 90 degrees (knob on probe should point toward the nose of mouse)
10. Lower probe on to mouse neck until image is obtained.
11. Manipulate stage left and right until trachea (mid-line structure) is identified
12. Identify left common carotid artery
13. Tilt imaging stage so that an angle is created between imaging plane and the carotid. The greater this angle, the stronger the Doppler signal will be.
14. Place pulse wave Doppler in middle of left common carotid.
15. Use angle correction as needed.
16. Repeat measurements on the right common carotid.
17. Successful partial carotid ligation will result in overall reduction in flow (~80-90%) in the left common carotid compared with the right side, with reversal of flow towards aortic inlet during diastole.
18. Once satisfactory images have been obtained, turn off anesthesia, wipe echo gel off mice and free mice from tape restraints. Place mice in warmed recovery chamber.

3. Intimal RNA isolation from carotid

1. Sacrifice mice by CO_2 inhalation according to the institutional IACUC protocol
2. Tape the paws to a paper towel.
3. Cut the skin of the mouse from the abdomen to the top of the thorax.
4. Open the abdominal wall below the ribcage with a sharp pair of scissors.
5. Lift the sternum with forceps and cut the diaphragm, then cut away the ribcage to expose the heart.
6. Cut the vena cava with scissors.
7. Pressure perfuse (120 mmHg) for 2 to 3 min with normal saline containing 10 Units/mL heparin through the left ventricle until the lungs and liver become pale.
8. Cut the skin of the neck and remove all the fat, muscles, and connective tissues until the carotid arteries are exposed.
9. Place the mouse under a dissecting microscope.
10. Puncture a hole right below the ligation sites in the left carotid for the second perfusion.
11. Pressure perfuse again for ~ 1 min with normal saline containing 10 Units/mL heparin through the left ventricle, making sure the left carotid is well perused.
12. Using a fine tip forceps and small spring scissors, carefully remove the peri-adventitial tissues surrounding the carotids. Be careful not to squeeze or stretch the carotids during this cleaning step.
13. Cut the left carotid artery between the aortic arch and the ligation points above the carotid bifurcation.
14. Cut the right carotid artery between the right subclavian artery branching point and the carotid bifurcation.
15. Transfer the carotids to a 35 mm culture dish, containing ice-cold HBSS. If needed, carefully remove any remaining peri-adventitial tissue.
16. Prepare an insulin syringe (3/10 ml syringe) with a 29g needle by filling with 150 µl QIAsol lysis buffer (Qiagen) per each carotid.
17. Carefully insert tip of needle into one end of the carotid.
18. While holding the carotid and the tip of the needle with a forceps, quickly (~ 1 sec) flush the QIAsol lysis buffer (150 µl) into a 1.5ml tube (intima eluate).
19. Rinse the carotid leftover (media+adventitia) once in HBSS, put it in a 1.5 ml tube, and snap-freeze in liquid nitrogen.
20. Intima eluate and the frozen leftover are then used for intimal or media+adventitia RNA isolation using the miRNeasy mini kit (QIAGEN) according to the manufacturer's instructions.

4. Representative Results:

We partially ligated mouse left carotid arteries and ultrasound examination was carried out one day following the procedure. As shown in Figure 1, successful partial ligation reduces blood flow velocity and reverses flow (disturbed flow) in the left common carotid artery during diastole. Partial ligation of ApoE knockout (KO) mice accompanied by a high fat diet induces robust atherosclerosis in the left common carotid artery(LCA), but not in the contralateral right carotid, by two weeks (Figure 2). RNA collected from the carotid intima contains endothelial marker genes such as PECAM-1, while free of medial smooth muscle cell marker genes such as a-SMA (Figure 3).

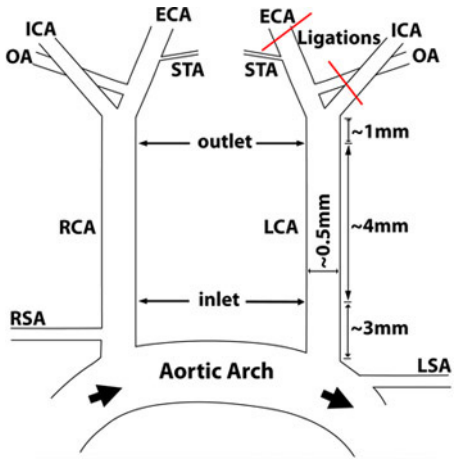

Figure 1: Schematic of carotid anatomy and ligations: Three branches of the left common carotid artery (LCA) [external carotid artery (ECA), internal carotid artery (ICA), and occipital artery (OA)] were ligated, while leaving the superior thyroid artery (STA) open.

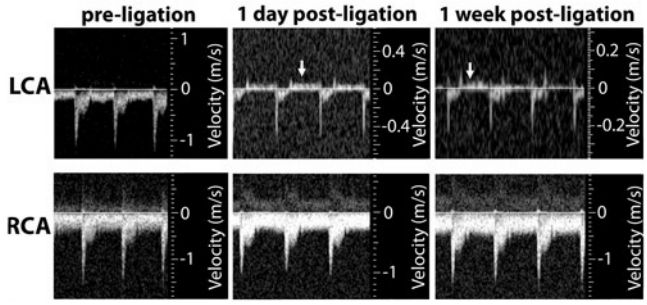

Figure 2: The ultrasound images show flow velocity profiles and reveal that partial ligation induces flow reversal (indicated by arrows) in LCA during diastole. Flow in RCA remains unchanged after ligation.

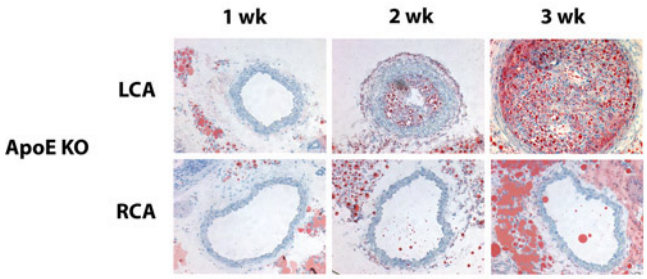

Figure 3: Partial ligation and high-fat diet rapidly induces atherosclerosis in LCA of ApoE KO mice. ApoE KO mice were partially ligated and fed the high-fat diet for 1 to 3 weeks. Frozen sections from LCA were stained with Oil-Red-O.

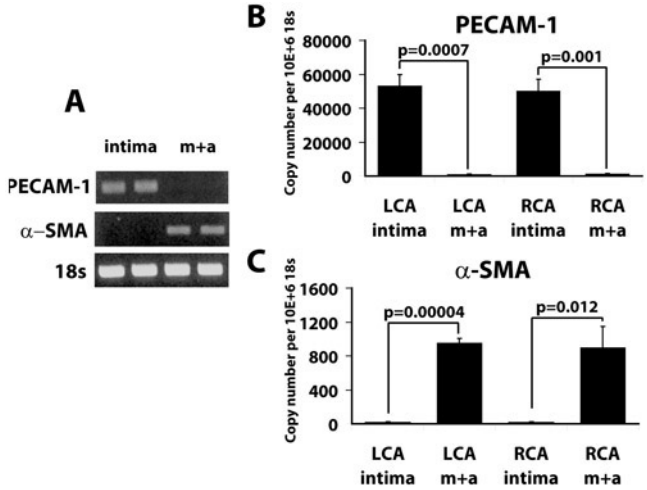

Figure 4. Method of intimal RNA preparation. Intimal RNA and medial+adventitial (m+a) RNA were obtained from sham-operated RCA and LCA in C57BL/6 mice. RNAs were analyzed by semi-quantitative RT-PCR **(A)** and qPCR **(B, C)** for PECAM-1 and α-SMA using 18s as an internal control. Bar graphs are mean ± SEM, n=3.

Discussion

In this procedure, proper identification of all the branches of the common carotid including the external carotid, internal carotid, occipital artery and superior thyroid artery is essential. It is also important to verify the flow pattern using ultrasound as demonstrated. In our experienced hands, our success rate of partial ligation surgeries based on the ultrasound studies of more than 500 mice is better than 90%. For the carotid intimal RNA preparations, it is important to pay attention to the appearance of the carotid after the first perfusion step. If there is evidence of flow blockage in the left common carotid at this point, we recommend not using these samples for further analysis.

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