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Molecular and physiological evaluation of pancreatic cancer-induced cachexia

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Abstract

Cachexia, a complex metabolic syndrome, is characterized by involuntary weight loss along with muscle wasting and fat depletion leading to poor quality of life of patients. About 80% pancreatic cancer patients exhibit cachectic phenotype at the time of diagnosis. Here, we present the several molecular and physiological parameters, which we utilize to study the pancreatic cancer-induced cachexia in in vitro models and pre-clinical mice models of pancreatic cancer. We have described myotube and adipocyte-based in vitro models of muscle and fat wasting, including methods of cell culture, differentiation, and treatment with cancer cell-conditioned medium. Furthermore, we have explained the methods of evaluation of key cachectic markers for muscles. Next, we have detailed the orthotopic implantation mouse models of pancreatic cancer and evaluation of different physiological parameters, including body weight, food intake, body composition analysis, glucose tolerance test, insulin resistance test, grip strength measurement, and rotarod performance test. We have also explained morphological parameters and molecular markers to evaluate the muscle wasting in pancreatic cancer-induced cachexia.

Keywords

Pancreatic cancer; Cachexia; Muscle wasting; C2C12; 3T3L1; Orthotopic implantation model; Fat depletion

1 Introduction

Cachexia is a devastating metabolic syndrome characterized by extreme weight loss due to muscle wasting with or without fat depletion [1]. Cachexia is related to several chronic

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diseases, and around 9 million people world-wide suffer from cachexia, including up to 80% of cancer patients [2]. Muscle wasting is a key hallmark of cancer-induced cachexia, which results in poor quality of life and reduced tolerance to therapy [3]. Cachexia also leads to declined physical functions, psychological stress, and increased mortality in cancer patients [4]. Energy imbalance, reduced food intake, and increased resting energy expenditure are key features of cachexia which play an important role in increased weight loss during cachexia [5]. Cancer cell metabolism and metabolic regulator genes play very important roles in pathogenesis and therapeutic responses in pancreatic cancer [6–10]. Recently, we have shown that metabolic reprogramming in cancer cells directly contributes to cancer-induced cachexia [11]. Bioactive compounds, including silibinin, can effectively prevent pancreatic cancer-induced cachexia [12]. Although the association between cancer and cachexia was known since the time of Hippocrates [13], till date, not a single drug is clinically approved for the treatment of cancer-induced cachexia. It is imperative to have a well-established system to evaluate the interventional therapeutic agents and study the novel regulators of pancreatic cancer induced-cachexia. Here, we have discussed the detailed procedures for in vitro and in vivo characterization of pancreatic cancer-induced cachexia.

2 Materials

1. C2C12 cells
2. 3T3L1 cells
3. S2–013 pancreatic cancer cell line
4. Insulin (10mg/ml)
5. Dexamethasone
6. Isobutylmethylxanthine
7. RNA isolation kit
8. cDNA synthesis kit
9. SYBR green master mix
10. Mouse Monoclonal Anti-Myosin Heavy Chain Antibody (MF20)
11. DMEM
12. Horse Serum
13. Fetal Bovine Serum
14. Nile Red
15. Collagen/gelatin-coated cell culture dishes/plates
16. Grip Strength Meter
17. Rotarod
18. Lunar PIXImus densitometer
19. Glucose meter and glucose measuring strips

20. Weighing balance
21. Anesthesia chamber with oxygen cylinder

3 Methods

3.1 Preparation of conditioned medium (CM) from pancreatic cancer cells

1. Seed the pancreatic cancer cells (e.g. S2–013) in a 100mm cell culture dishes with 10% FBS-containing DMEM.
2. When cells become 80% confluent, wash the cells with PBS, and add 10 ml serum-free DMEM.
3. After 24h collect the medium, spin at 2500rpm for 5min, filter sterilize, and store at -80°C until further use.

3.2 Differentiation of C2C12 cells into myotubes

1. Seed the C2C12 cells in collagen-coated dishes in 10% FBS-containing DMEM (see Note 1).
2. When cells become confluent, wash the cells twice with the PBS and add differentiation medium (DMEM + 2% horse serum+ insulin $1\mu\text{g/ml}$).
3. Differentiate the cells for 72h to 96h.
4. Change the differentiation medium every 24h initially (up to 48h) and every 12h afterward (see Note 2).
5. Monitor the morphology of differentiated myotubes using microscopy. Figure 1 shows representative images of C2C12 myoblasts and differentiated myotubes.

3.4 Treating differentiated myotubes with cancer cell CM

1. Reconstitute cancer cell CM by adding 2% horse serum and $1\mu\text{g/ml}$ insulin. Warm the conditioned medium as well as the control medium at 37°C .
2. Aspirate the media from differentiated myotubes and add the cancer cell CM along with the control medium.
3. Change the CM every 24h (see Note 3).
4. Treat the differentiated myotubes with CM for 24h to 72h.
5. At the end of the treatment, capture bright-field images of myotubes and harvest the samples as needed (see Note 4).

3.6 Evaluation of myotube degradation and CM-induced proteolysis

1. After conditioned-medium treatment, capture the bright-field images of control and CM-treated myotubes.
2. Representative images of different treatments can be presented.

3. Average myotube diameter can be measured by ImageJ software and presented as a bar-chart.
4. To evaluate the proteolysis induced by CM, harvest the cells in cell lysis buffer and quantify the total protein content by the Bradford method. Relative protein quantity can be presented as a bar-chart.

3.7 Evaluation of cachectic markers by qRT-PCR

1. Isolate the total RNA from control and CM-treated myotubes by using the standard procedure.
2. Synthesize the cDNA by utilizing commercially available kits.
3. Evaluate the expression of *Trim63* (MuRF1) and *Fbxo32* (Atrogin1) in control and CM-treated samples by qRT-PCR (see Note 5).
4. Setup the qPCR reaction for each set of primes as follows:

cDNA	3µl
Forward primer(1µM)	1µl
Reverse primer (1µM)	1µl
SYBR green master mix (2X)	5µl

5. Setup the thermal cycle conditions as follows:
95°C 10min (1 cycle)
95°C for 10s and 60°C for 60s (40 cycles)
6. Calculate the relative expression of *Trim63* and *Fbxo32* by the Ct method [14]. Use *Actb* (β-actin) as an internal control (see Note 6).

3.8 Evaluation of Myosin Heavy Chain (MyHC) degradation by immunoblotting

1. Extract the protein from control and CM-treated myotubes by utilizing cell lysis buffer with protease inhibitors.
2. Load 5–10µg of total protein on 8% SDS-PAGE gel and run the gel until the dye front runs off the bottom of the gel.
3. After transferring the protein onto the nitrocellulose membrane, block the membrane by incubating in 5% skimmed milk in PBST (PBS with 0.1% Tween-20) for 1h.
4. Incubate the membrane with monoclonal MyHC antibody in 5% milk prepared in PBST overnight at 4°C. Use 1:500 – 1:1000 dilution of antibody.
5. Next day, wash the blot three times with PBST and incubate with anti-mouse HRP-conjugated secondary antibody for 45min.
6. Wash the blot three times with PBST and develop the blot by using the ECL Chemiluminescent Substrate Reagent Kit.
7. Use β-actin as the loading control.

3.9 Differentiation of 3T3L1 cells into adipocytes

1. Seed 3T3L1 cells in 10% FBS containing DMEM (see Notes 7).
2. When cells become confluent, wash twice with PBS and add differentiation medium (DMEM containing 10% FBS, 1 μ M dexamethasone, 0.5mM isobutylmethylxanthine /IBMX, and 10 μ g/mL insulin).
3. Culture the cells for two days in differentiation medium. Change the differentiation medium after every 24h.
4. After 2 days, replace the differentiation medium with maintenance medium (DMEM with 10% FBS and 10 μ g/mL insulin). Change the maintenance medium after every 48h.
5. When fat globules become visible after 3–4 days (Figure 2), replace the maintenance medium with normal culture medium (DMEM with 10% FBS).

3.10 Evaluation of CM-induced adipolysis by Nile red staining

1. Reconstitute CM by adding 10% FBS. Warm the CM as well as the control medium at 37°C.
2. Aspirate the differentiation medium from the differentiated myotubes and add the cancer cell CM along with the control medium.
3. Change the CM after every 24h.
4. Treat the differentiated adipocytes with the cancer cell CM for 48h to 72h.
5. At the end of the treatment, wash cells two times with PBS and Nile red solution (1 μ g/ml in PBS) (see Note 8).
6. Incubate the cells with Nile red solution at 37°C for 30min, protected from light.
7. Wash the cells two times with PBS and capture the fluorescent images by using a fluorescent microscope. Figure 2 shows a representative image of Nile red stained differentiated adipocytes. Representative images of different conditions can be presented.
8. Fluorescent intensity can be measured by utilizing imaging software and represented as a bar diagram.

3.11 Evaluation of adipolysis markers by qRT-PCR

1. Isolate the total RNA from control and CM-treated adipocytes by using the standard procedure.
2. Synthesize the cDNA by utilizing commercially available kits.
3. Evaluate the expression of *Azgp1* (zinc alpha-2-glycoprotein) and *Lipe* (hormone sensitive lipase) in control and CM-treated samples by qRT-PCR (see Note 9).
4. Setup the qPCR reaction for each set of primes as follow:

cDNA

3 μ l

Forward primer (1μM)	1μl
Reverse primer (1μM)	1μl
SYBR green master mix (2X)	5μl

5. Setup the thermal cycle conditions as follows:
95°C 10min (1 cycle)
95°C for 10s and 60°C for 60s (40 cycles)
6. Calculate the relative expression of *Azgp1* and *Lipe* by the Ct method [14].
Use *Actb* (β-actin) as an internal control.

3.12 Orthotopic implantation mouse model of pancreatic cancer

1. Anesthetize the mice by utilizing injectable anesthesia agent or by using an anesthesia chamber.
2. Prepare the mice (athymic nude) for implantation by applying betadine solution three times at the incision site followed by wiping with alcohol wipes three-times.
3. Make incisions in the skin and the peritoneum on the lateral side of the mice by utilizing sterile scissors.
4. Inject 0.5×10^6 S2–013 cells with a 27G needle syringe into the pancreas of mice. Suture the peritoneum with chromic gut sutures and the skin with nylon sutures.
5. Inject 100μl sterile saline solution subcutaneously into mice.
6. Place mice in a fresh cage on a heat-pad till they become fully active (see Note 10).
7. After 5–7 days of implantation, start evaluating all the physiological parameters.

3.13. Body weight measurement of mice

1. Prepare a weighing scale with a sanitized plastic bowl/beaker.
2. Tare value on the scale to zero before each measurement.
3. Place mouse in the vessel and allow the mouse to settle. Record the weight that appears to be a steady state.
4. Sanitize the plastic bowl/beaker between each mouse.
5. Record body weight every two days at the same time of the day (see Note 11).
6. Plot the progressive body weight at the end of the study.

3.14 Food intake measurement of mice

1. Prepare a weighing scale with a sanitized plastic bowl/beaker.
2. Tare the displayed value on the scale to zero before each measurement.

3. Measure the food from a mouse cage and record value. If necessary, add more food and record new weight before replacing food in the cage.
4. Calculate the food intake by determining the change of food weight between weekly measurements at the same time of the day. Divide the total change in food weight by the number of mice in the cage and days between measurements to generate a value for food intake per mouse per day.
5. Calculate food intake at the same time of the day between longitudinal measurements (see Note 11).
6. Cumulative food intake can be plotted at each time point of the study on the basis of data collected by the method mentioned above.

3.15 Evaluation of forelimb grip strength

1. Prepare grip strength meter and set the apparatus to record the maximal force in a unit of your choice.
2. Acclimatize mice to procedure room for 15 minutes before assessment of grip strength.
3. Reset the displayed value on the grip strength meter to zero before each measurement.
4. Obtain a mouse from its cage. Grip the base of its tail between your thumb and the index finger, and lower the mouse over the pull grid of the grip strength meter such that only the front paws can grasp the grid (see Note 12)
5. Permit the mouse to properly grab the grid before pulling the mouse away from the grip strength meter.
6. Maintain the torso of the mouse in a horizontal position while you pull the mouse away in a fluid and controlled motion. Continue to pull the mouse horizontally until the mouse releases the pull grid.
7. Record the maximal grip strength, repeat until three total measurements are recorded, and use the average of the measurements for analysis.
8. Sanitize the pull grid with 70% ethanol between measurements of different mice cages. Allow the apparatus to dry completely before further measurements.
9. Evaluate grip strength at the same time of the day between longitudinal measurements (see Note 11).

3.16 Assessment of motor coordination with a rotarod apparatus

1. Prepare a clean rotarod apparatus and place a padded object in every exercise lane to cushion the fall of the mouse. Generate a program to begin spinning at three rpm and increasing one rpm for every subsequent ten seconds. Keep these parameters constant for the whole study.
2. Acclimatize the mice to procedure room for 15 minutes before starting the rotarod testing.

3. Place mouse on rotarod so that they walk forward and against the spin of the apparatus. Allow mice to familiarize themselves with walking against a rod rotating at three RPM for one minute.
4. Begin the rotarod testing by activating the program and record the latency of fall.
5. Complete three total trials with ten minutes of rest between each replicate. Use the average of the recorded values for further analysis.
6. Sanitize the apparatus with 70% ethanol between experiments with different mice.
7. Measure latency of fall at the same time of the day between longitudinal measurements (see Note 11).

3.17 Dual-energy X-ray absorptiometry of mice

1. Switch on the Lunar PIXImus densitometer (GE Medical-Lunar, Madison, WI).
2. Initialize the PIXImus software and perform the quality control measurement by using the phantom mice.
3. Anesthetize the mice in an anesthesia chamber supplied with oxygen. Monitor the mice until they are adequately sedated.
4. Weigh the mouse and measure the length of the mouse. Following is the method to accurately measure the length of the mouse:
 - a. Place the mouse on a disinfected ruler.
 - b. Gently press the head against the ruler and pull its tail to straighten its spine.
 - c. Measure the mouse from the nostril to the beginning of its tail.
5. Now place the mouse on the Lunar PIXImus densitometer platform and perform a scout scan (see Note 13).
6. Select the area of interest and perform a measurement scan.
7. Record the bone mineral content (BMC), bone mineral density (BMD), lean body mass, total fat, and percentage of fat.
8. After measurement, remove the mouse from the platform and set it on a 37°C heat pad and monitor until it gains consciousness.
9. Return the mice to their respective cages.
10. Data obtained from the above process can be utilized to demonstrate fat depletion during the progression of cancer-induced cachexia.

3.18 Intraperitoneal Glucose Tolerance Test (IPGTT)

1. Fast mice overnight or at least for 16h. Ensure that the mice have access to drinking water at all times.

2. Prepare a 20% glucose solution and then filter sterilize it using a 0.22µm syringe filter.
3. Weigh mice and calculate the amount of glucose solution needed for each mouse at a concentration of 2g/kg body mass.
4. Pick up a mouse and massage its tail to increase circulation. Collect the blood by tail vein incision with a sterile scalpel blade and let a drop of blood (approximately 5µl) fall into the glucose-measuring strip. This blood glucose measurement is going to be the baseline and will serve as t=0 time point.
5. Sterilize the abdominal region of the mouse using 70% ethanol. Inject the required calculated amount of the glucose solution intraperitoneally (see Note 14).
6. Now measure the blood glucose after 15, 30, 60, 90, and 120min.
7. Plot the blood glucose level at different time points.
8. Monitor the mouse for at least 2h for any abnormal behavior.

3.19 Insulin Resistance Test

1. Fast the mice for just 4 h. The food can be removed early in the morning at 7 am.
2. Similar to the glucose tolerance test, take glucose measurements at zero time point, before injecting glucose. Collect a drop of blood by tail vein incision tail for blood glucose measurement as described for the glucose tolerance test.
3. Inject the mouse with 0.5 U/kg glucose intraperitoneally with the 27G needle (see Note 14).
4. Take blood glucose measurements at 15, 30, 60, 90, and 120min.
5. Plot the blood glucose level at different time points.
6. Monitor the mouse at least 2h for any abnormal behavior.

3.20 Post-Necropsy Measurements

At the end of the experiment, mice are euthanized, and organs are harvested and frozen or fixed as needed for the future applications. Following are the post-necropsy measurements that assist in determining the cancer-induced cachexia in mice:

1. *Body weight:* Each mouse should be weighed after euthanasia. This should be done before blood or tissue collection.
2. *Carcass Weight:* Weight of each mice should be taken after the tumor is removed from the body. Make sure not to take out any other organs before measuring the carcass weight.
3. *Dry Heart Weight:* Heart is taken out of the body cavity and washed in saline. Wash until most of the blood has drained. Then place in a 37°C incubator for 4 to 6h. After that, weigh the dry heart.

4. *Muscle weight:* Carefully pin down the hind legs of the mice. With a pair of sharp scissors, cut the skin off from the muscles of the hind legs. Wash the scissors to get any mouse hair off of them. Carefully cut out the gastrocnemius muscle (the larger calf muscle), tibialis anterior, and quadriceps from the leg and place the muscle on a weighing balance. After weighing, wrap the muscle in an aluminum foil and stored at -80°C for further analysis. Fix one part of the muscle in methacorn for the immunohistochemical studies.
5. *Fat weight:* White fat is taken from the abdomen region of the mice and weighed on the balance.
6. Tumor weight and tumor volume should be recorded at the time of necropsy (see Note 15)

3.21 Morphological analysis of muscle

1. Paraffin embed the fixed muscle tissues and prepare $5\mu\text{m}$ thick sections of the muscles with the help of a microtome.
2. Stain the muscle sections with hemotoxylin and eosin (H&E) stain and capture the bright field images of different representative areas (see Note 16).
3. Using the ImageJ software calculate the cross-sectional area of each muscle fiber in at least three representative images.
4. Plot the average cross-sectional area of the muscle fibers between control and cancer-bearing mouse muscles.

3.22 Evaluation of cachectic markers in muscle

1. Prepare the protein lysate from the frozen muscle tissues of mice (see Note 17).
2. Perform the MyHC expression by immunoblotting as described in section 3.8.
3. Utilize beta-actin immunoblot as a loading control.
4. Isolate the RNA from frozen muscle tissues by utilizing standard RNA isolation procedures.
5. Synthesize the cDNA by utilizing cDNA synthesis kit.
6. Evaluate the expression of *Trim63* and *Fbxo32* by utilizing qRT-PCR as described in section 3.7.
7. Use the *Actb* for normalizing the expression of different genes.

4 Notes

1. Never allow the C2C12 cells to become confluent. In normal tissue culture dishes, myotubes start coming off some time after differentiation. Gelatin coated dishes can also be used.

2. Differentiating myotubes consume the media component very fast and secrete a lot of lactic acid, so it is very important to change the medium regularly to avoid stress or death of myotubes.
3. Change the CM regularly to avoid premature death of myotubes. Be very careful when you remove the medium from plates, never let them dry completely.
4. For protein extraction, harvest myotubes in protein lysis buffer with protease inhibitors and for RNA isolation directly harvest myotubes in the Trizol reagent. Store at -80°C .
5. Primer sequences of mouse *Trim63* and *Fbxo32* genes are as follows:
Fbxo32-F TGAGCGACCTCAGCAGTTAC
Fbxo32-R GCGCTCCTTCGTA CTTTCCTT
Trim63-F AGGACAACCTCGTGCCTACAAG
Trim63-R ACAACCTGTGCCGCAAGTG
6. Primer sequences of mouse β -*Actin* gene are as follows:
Actb-F CTGTCCCTGTATGCCTCTG
Actb-R ATGTCACGCACGATTTC
7. Never allow 3T3L1 cells to get confluent.
8. The stock solution of Nile Red stain should be prepared in acetone. We prepare 1mg/ml solution and dilute it 1000 times in PBS for further use. Protect the stock solution from light.
9. Primer sequences of *ZAG1* and *HSL* genes are as following:
Azgp1-F GAGCCTGTGGGACCTTGGA
Azgp1-R CCTCCCTGGCCCTCTGAA
Lipe-F GGCTTACTGGGCACAGATACCT
Lipe-R CTGAAGGCTCTGAGTTGCTCAA
10. Keep heat pad temperature low and regularly monitor the breathing by mice. All the procedures should be performed according to the institutional IACUC guidelines.
11. Measuring mouse parameters at the same time of the day limits confounding variables resulting from eating, sleeping, and activity habits.
12. Do not permit the mice to grab the metal mesh with both limbs (anterior and posterior).
13. Place the mouse on the platform at the almost similar place to avoid a variation in measurements. The mouse should be completely anesthetized, and there should be no movement during the measurement process.

14. While injecting the mice, make sure to go into the intraperitoneal cavity at a shallow angle. Going straight in will puncture the inferior vena cava and going too shallow will create a bubble under the skin.
15. All the tissues should be flash frozen to avoid protein, RNA, or metabolite degradation.
16. During the preparation of tissue sections, cross sections of muscle tissues should be prepared.
17. It is difficult to do complete protein extraction from the muscle tissues. We suggest performing light sonication for protein isolation from the muscles.

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Figure 1:

A. Undifferentiated C2C12 cells (confluent after 48h of seeding). B. Differentiated C2C12 cells, after 72h of differentiation.

Figure 2:

A. Undifferentiated 3T3L1 cells. B. Differentiated 3T3L1 with fat globules. C. Nile red-stained differentiated 3T3L1 cells.