Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - Author Produced Video
Manuscript Number:	JoVE58688R2
Full Title:	Evaluation of the interplay between the complement protein C1q and hyaluronic acid in promoting cell adhesion.
Keywords:	complement system; C1q; extra cellular matrix; hyaluronic acid; cell adhesion; tumor cells
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TITLE:

Evaluation of the interplay between the complement protein C1q and hyaluronic acid in promoting cell adhesion

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KEYWORDS:

Complement System, C1q, Extracellular Matrix, Hyaluronic Acid, Cell adhesion, Tumor Cells

SUMMARY:

The complement component C1q is a pro-inflammatory molecule highly expressed in the tissue microenvironment that can interact with the extracellular matrix. Here, we described a method to test how C1q bound to hyaluronic acid impacts cell adhesion.

ABSTRACT:

It has been increasingly demonstrated that the tumor microenvironment plays an active role in neoplasia growth and metastasis. Through different pathways, tumor cells can efficiently recruit stromal, immune and endothelial cells by secreting stimulatory factors, chemokines and cytokines. In turn, these cells can alter the signaling properties of the microenvironment by releasing growth-promoting signals, metabolites and extracellular matrix components to sustain high proliferation and metastatic competence. In this context, we identify that the complement component C1q, highly expressed locally by a range of human malignant tumors, upon interacting with the extracellular matrix hyaluronic acid, strongly affects the behavior of primary cells isolated from human tumor specimens. Here, we described a method to test how C1q bound to hyaluronic acid (HA) impacts tumor cell adhesion, underlying the fact that the biological properties of key components of the extracellular matrix (in this case HA) can be shaped by bioactive signals toward tumor progression.

INTRODUCTION:

The tumor microenvironment (TME) influences cancer development and progression since it can provide a permissive niche for cell survival, growth and invasion. The identification of new key players in TME may be useful for the discovery of new molecular tools for target therapy. TME includes a complex and dynamic network of non-malignant cells, such as endothelial cells, fibroblasts and cells of the immune system, embedded in the surrounding extracellular matrix (ECM) components including collagens, laminins, fibronectins, proteoglycans and hyaluronans. Both tumor and non-tumor cells synthesize and secrete ECM components together with cytokines, chemokines, growth factors and inflammatory and matrix remodeling enzymes that overall alter the physical, chemical and signaling properties of TME. Among these constituents, hyaluronic acid (HA) has emerged to exert a crucial role in tumor biology. Despite its simple chemical composition, HA, together with its HA-binding molecules (hyaladherins), can modulate angiogenesis, immune system responsiveness and ECM remodeling in a size and concentration dependent manner[1].

The complement (C) system is also part of the local TME, which has recently received increasing attention. The C system encompasses a set of soluble and membrane-bound proteins involved in the first line of defense against non-self-cells, unwanted host elements and pathogens. Functionally, the C links the two-effector arms of innate and adaptive systems to promote either direct cell killing or mounting of an inflammatory response[2]. C activation can suppress tumor growth, by destroying cancer cells or inhibiting their outgrowth, but it has become increasingly clear that it can possess a tumor-promoting activity by sustaining chronic inflammation, promoting the establishment of an immunosuppressive milieu, inducing angiogenesis, and activating cancer-related signaling pathways[3]. In this context, C1q, the first recognition molecule of the classical pathway of the C system has emerged to exert important functions in the tumor microenvironment independently of C activation[4]. C1q has been shown to be expressed locally by a range of human malignant tumors, where it can favor cancer cell adhesion, migration and proliferation in addition to angiogenesis and metastasis[5]. Interestingly C1q interacts to a major constituent of the ECM such as HA.

We developed a technique to isolate the primary cancer cells from the tumor mass. Furthermore, we create the matrix, which can stimulate tumor microenvironment, particularly the interaction between C1q and high molecular weight hyaluronic acid. C1q bound to HA was able to induce adhesion of the tumor cells.

PROTOCOL:

Tissue samples from patients were collected after informed consent following approval of the ethical considerations by the Institutional Board of the University Hospital of Trieste, Italy.

1. Tumor cell isolation and culture (Day 1)

1.1. Isolate human mesothelioma cells from MPM solid specimens. Finely mince the tissue with a cutter to obtain fragments of about 2-3 mm^2 in size and incubate in 5 mL of digestion solution composed of Hanks' Balanced Salt Solution (HBSS) supplemented with 0.5 mM Ca^{2+}/Mg^{2+}, 0.5% trypsin and 50 µg/mL DNase I, overnight at 4 °C.

1.2. Tumor cell isolation and culture (Day 2)

1.2.1. After overnight incubation, place the digested tissue for 30 min in a 37 °C incubator with

gentle shaking.

1.2.2. Replace the digestion solution upon centrifugation (250 x *g*) with 3 mg/mL collagenase type 1 dissolved in 5 mL of Medium 199 with HBSS and further incubate for 30 min at 37 °C with gentle shaking.

1.2.3. Block the enzymatic reaction by adding 10% heat-inactivated fetal bovine serum (FBS). Resuspend the cells very carefully with a 5 mL pipet to ensure that most of the cells are released from the tissue. Then, filter the cell suspension through a 100 µm pore filter.

1.2.4. Seed the cells in a 12.5 cm^2 flask and culture them at 37 °C with human endothelial cells serum-free medium (HESF), with 10% FBS and supplemented with EGF (10 ng/mL), basic FGF (20 ng/mL), and 1% penicillin– streptomycin.

NOTE: Replace with fresh medium every 2–3 days.

2. HA coating (Day 1)

2.1. Resuspend high molecular weight HA (1.5 kDa) in double distilled water at the concentration of 1 mg/mL[6].

2.2. Dilute HA stock solution to 50 µg/mL in carbonate/bicarbonate buffer (0.1 M, pH 9.6) with gently pipetting.

2.3. Coat the 96-well plate with 100 µL of dilute HA stock solution per well overnight at 4 °C.

NOTE: Hyaluronic acid was a kind gift from Professor Ivan Donati, Department of Life Sciences, University of Trieste[7].

3. C1q coating (Day 2)

3.1. After overnight incubation, vacuum aspirate the treated wells and wash the 96-well plate with 100 µL of Dulbecco's PBS (dPBS) per well.

3.2. Allow C1q (25 µg/mL or different concentrations for dose response experiments) or BSA (as a negative control) to bind to plastic immobilized-HA by incubating these proteins at a concentration of 25 µg/mL in 100 µL of dPBS + 0.5% BSA and 0.7 mM Ca^{2+}/Mg^{2+}. Then incubate overnight at 4 °C.

3.3. Vacuum aspirate the wells and wash the 96-well plate with 100 µL/well of dPBS.

4. Cell labeling with FAST DiI

4.1. Resuspend 1 x 10^5 tumor cells in 500 µL of dPBS containing 10 µg/mL of the fluorescent dye FAST DiI. Incubate for 15 min at 37 °C in a 5% v/v CO_2 incubator for the labelling, mixing manually after 5 min intervals.

4.1.1. To remove excess FAST DiI, add 10 mL of dPBS, pipette gently up and down, and centrifuge at 250 x *g* for 7 min. Resuspend the cell pellet in 1 mL of human endothelial serum

free medium containing 0.1% BSA (HESF + 0.1% BSA).

5. Cell Adhesion on HA/C1q matrices (Day 1)

5.1. Vacuum aspirate the wells coated with the different matrixes (wells were coated in step 3.2).

5.2. Distribute 100 µL of the labelled cell suspension to the coated wells and incubate the plate for 35 min at 37 °C in 5% v/v CO_2 incubator.

5.3. Remove the non-adherent cells by aspirating the supernatant. Wash once with dPBS containing 0.5% BSA, 0.7 mM Ca^{2+} and 0.7 mM Mg^{2+}.

5.4. Lyse the adherent cells by adding 200 µL/well of 10 mM Tris–HCl, pH 7.4 + 0.1% v/v SDS and immediately read the 96-well plate with a fluorescence reader (544 nm, emission 590 nm) using a calibration curve generated through the lysis of an increasing number of labelled cells.

REPRESENTATIVE RESULTS:

HA is a negatively charged high-molecular-weight polysaccharide, which is made up of repeating (β,1–4)-D-glucuronic acid-(β,1–3)-N-acetyl-D-glucosamine disaccharide units (**Figure 1B**)[7]. The occurrence of the binding of HA on the 96-well plate as well as the efficiency of its immobilization were tested taking advantage of biotinylated HA (bio-HA). Different concentrations of Bio-HA, ranging from 10 µg/mL to 1 mg/mL, were re-suspended in 100 mM carbonate/bicarbonate-buffer pH 9.6 and incubated overnight at 4 °C.

After extensive washes, bio-HA bound to 96-wells plate was detected using streptavidin conjugated to alkaline phosphatase, while the presence of streptavidin was quantified using pNPP (1 mg/mL) as a substrate. The reading was performed at the wavelength of 405 nm using an ELISA reader. Bio-HA was able to bind to a 96-well plate in a dose response manner (data not shown); 50 µg/mL was chosen as a saturation plateau and therefore used in our assays.

Our previous data demonstrated that C1q is able to bind to a wide range of target ligands localized in the ECM[8]. This binding is particularly strong with HA[9]. The wells of a microtiter plate were coated with 50 µg/mL HMW-HA and incubated with increasing concentration of C1q. Bound C1q was shown to be incubating in the wells with anti-C1q polyclonal antibodies. C1q is able to bind to high molecular weight HA in a dose response manner (**Figure 1A**), reaching the maximum level of binding at the concentration of 50 µg/mL. Having established that C1q can bind to HA, we investigated the implication of this interaction in modifying the signaling properties of the ECM and their implications in tumor development. To this aim, we established a protocol to isolated tumor cells from bioptic specimens obtained from affected patients. Primary tumor cells were isolated from biopsy tissue, as summarized in **Figure 2**. The capability of tumor cells to interact with C1q was evaluated by performing a cell adhesion assay using different matrix combinations, as shown in **Figure 3**. Tumor cells were stained with the fluorescent probe FAST DiI and seeded onto immobilized HA, HA bound to C1q or to BSA (used as a negative control), for 35 min. If compared to HA, the coating with HA-bound-C1q was able to increase the amount of adhering primary cells but is not able to stimulate the adhesion of tumor cell lines that we tested, as shown in the representative graph in **Figure 3**.

FIGURE AND TABLE LEGENDS:

Figure 1. Interaction of C1q with hyaluronic acid. (**A**) Binding of C1q on HMW HA in a dose dependent manner. The data are expressed as the mean of three independent experiments in triplicate ± S.E.M. (**B**) Schematic representation of the C1q molecule and of the recombinant single chain globular region. C1q is assembled from three polypeptide chains (**A, B, C**): each containing an N-terminal collagen-like sequence and a C-terminal globular gC1q head. (**C**) Chemical formula of hyaluronic acid, a highly polymerized chain of glucuronic acid and N-acetylglucosamine.

Figure 2. Summary scheme of the isolation and morphology of tumor cells. Tumor cells were isolated from pleural biopsy specimens. The cells were seeded in a 12.5 cm^2 flask and cultured in Human Endothelial Cell Serum Free Medium + 10% FBS.

Figure 3. Effect of C1q on tumor cells adhesion. Tumor cells (MES) or primary mesothelioma cell line (MSTO-211H) were seeded to microtiter wells pre-coated with hyaluronic acid (HA), HA bound to C1q or to bovine serum albumin (BSA). In the upper part of the figure, the morphological aspect of one representative primary cell line adhered to HA, HA bound to C1q or to BSA was shown. Images were acquired via phase-contrast microscope, original magnification: 200x. FAST DiI labelled primary mesothelioma cells or a mesothelioma cell line (MSTO-211H) were allowed to adhere to microtiter wells pre-coated with HA, HA bound to C1q or to BSA. The data are expressed as mean of three independent experiments done in triplicates ± S.E.M. * $p < 0.01$ vs HA.

DISCUSSION:

We described an easy method to investigate how the complement component C1q, interacting with hyaluronic acid, is able to modulate the behavior of primary cells isolated from human tumor tissues. Both HA and C1q are abundantly present in the tissue microenvironment both under physiological and pathological conditions, participating to several cell biological processes. For instance, C1q has been shown to be present in the microenvironment of the placenta where it favors extravillous trophoblast invasion of the maternal decidua during placentation[8]. C1q is also deposited in wound-healing skin where it fosters the angiogenetic process required for tissue regeneration and repair[10]. Finally, C1q has been identified in several different tumor tissues[4]. High molecular weight HA is a natural barrier for angiogenesis and proliferation and recent evidence showed that C1q, besides its classical role in complement activation, is able to act as an ECM molecule, favoring cell migration.

The novelty of this work consists in the finding that the binding of C1q to HA strongly modify the interaction of tumor cells with HA. To set up this method several checkpoints have been considered. First of all, we ensured that a sufficient amount of HA was bound to the plastic of culture and ELISA wells using biotinylated HA. The Alcian blue staining of the wells confirmed these results (data not shown). The incubation has been performed overnight in bicarbonate buffer, pH 9.6, and this procedure ensure the complete saturation of the wells by HA (data not shown).

The second step was to bind C1q to HA. The binding of C1q to HA has been performed at a physiological pH (7.4) and in the presence of Ca^{2+}. For this reason, we used a dPBS-BSA buffer with bivalent ions. We initially performed a dose response experiment in order to identify and choose the optimal amount of C1q that can be found attached to HA. Although in our dose-response curve we did not reach exactly the plateau, we used the concentration of 25 µg/mL to be closer to the physiological concentration of free C1q, considering that the amount of C1q normally present in the serum is 75-150 µg/mL. It has been calculated that serum C1q free from C1r and C1s is about 10%-20% of C1 complex[11]. C1q is known for its ability to bind polyanions,

it has been defined as a charge pattern recognition molecule and HA is a negatively charged linear polymer of repeating units of (β,1–4)-D-glucuronic acid-(β,1–3)-N-acetyl-D-glucosamine[12,13]. For this reason, we investigated this strong non-covalent interaction. Our observations indicated that tumor cells sense the difference between HA and HA bound to C1q. The cells appear to be more spread-out if compared to the cell adhering to HA alone. We observed that this effect is not mediated by soluble C1q, indicating that this modification in cell adhesion is dependent on the interaction of C1q with HA, probably due to a conformational change of the complement molecule as a consequence of the binding.

We want to emphasize the importance of the use of primary cells as in vitro models of tumor behavior for the adhesion experiments, since we notice a strong difference in the adhesion capability between primary cells compared to several cell lines, as shown in **Figure 3**. An alternative and very interesting model to evaluate the biological properties of HA is the use of 3D models of HA hydrogel scaffold. This scaffold with the incorporation of biological molecules can be used for the evaluation of bioactive signals and can be used for several applications in the regenerative medicine[14]. The model that we proposed in this study is an easier, faster and cheaper method for the evaluation of cellular response to a combination of stimuli that can be considered a first step for the understanding of the molecular mechanism involved in this kind of interaction.

ACKNOWLEDGMENTS:

We thank Ivan Donati for providing of HA, Leonardo Amadio, Gabriella Zito (Department of Gynaecology of IRCCS “Burlo Garofolo”, Trieste, Italy) and Andrea Romano (Operative Clinical Unit of Anatomy and Pathological Histology, Cattinara Hospital, Trieste, Italy) for the tissue sample collection. We thank also Nicolò Morosini for the help in the video preparation and Alex Coppola, the voice. This work was supported by grants from the Institute for Maternal and Child Health, IRCCS “Burlo Garofolo”, Trieste, Italy (RC20/16) and Fondazione Cassa di Risparmio Trieste to R.Bulla.

DISCLOSURES:

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES:

1 Zamboni, F., Vieira, S., Reis, R. L., Oliveira, J. M., Collins, M. N. The potential of hyaluronic acid in immunoprotection and immunomodulation: Chemistry, processing and function. *Progress in Materials Science*. **97,** 97-122 (2018).

2 Lorusso, G., Ruegg, C. The tumor microenvironment and its contribution to tumor evolution toward metastasis. *Histochemistry and Cell Biology*. **130** (6), 1091-1103 (2008).

3 Ricklin, D., Hajishengallis, G., Yang, K., Lambris, J. D. Complement: a key system for immune surveillance and homeostasis. *Nature Immunology*. **11** (9), 785-797 (2010).

4 Bulla, R. et al. C1q acts in the tumour microenvironment as a cancer-promoting factor independently of complement activation. *Nature Communications.* **7**, 10346 (2016).

5 Winslow, S., Leandersson, K., Edsjo, A., Larsson, C. Prognostic stromal gene signatures in breast cancer. *Breast Cancer Research.* **17,** 23 (2015).

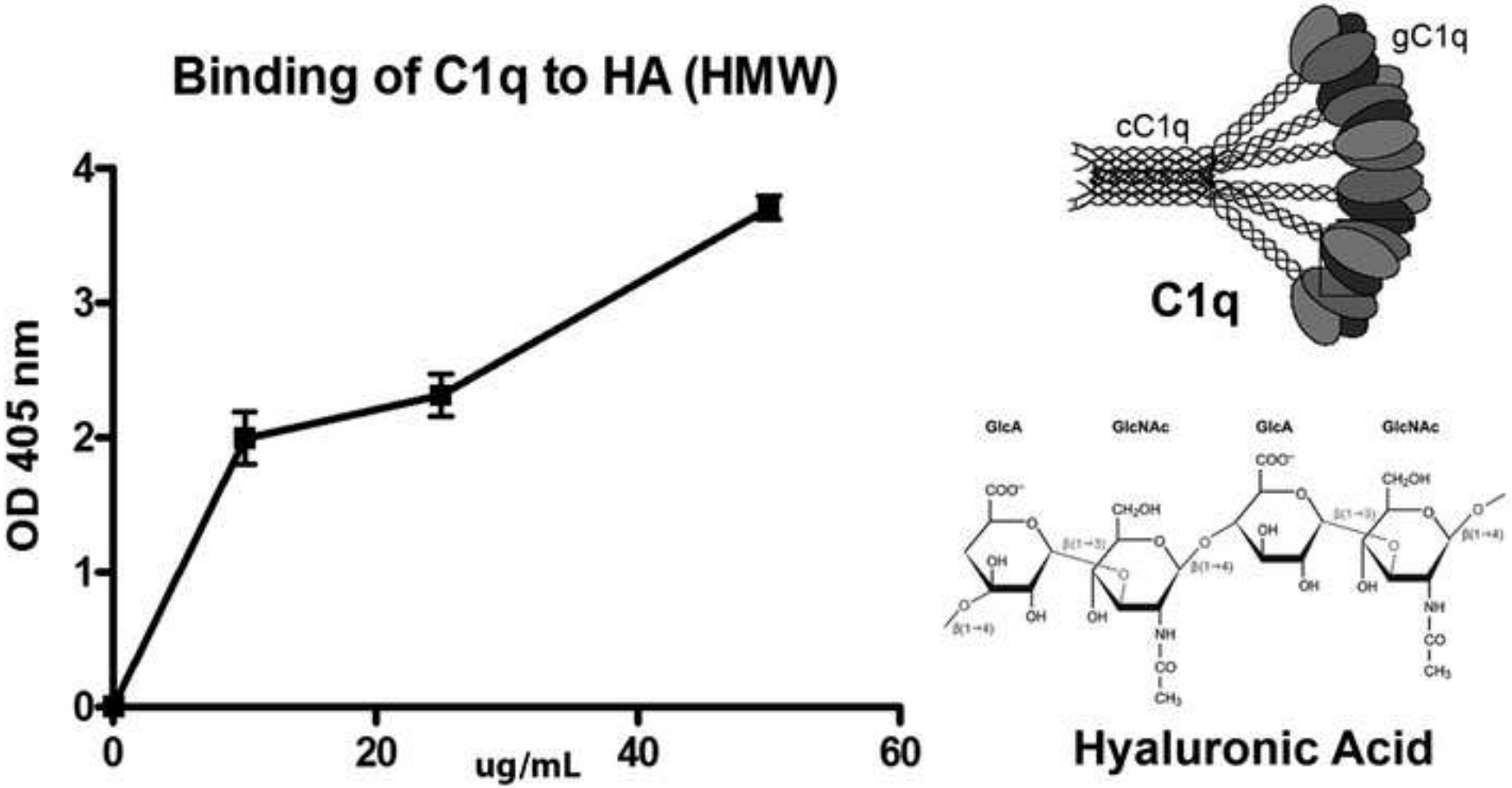
6 Collins, M. N., Birkinshaw, C. Hyaluronic acid solutions: A processing method for efficient chemical modification. *Journal of Applied Polymer Science.* **130** (1), 145-152 (2013).

7 Itano, N. Simple primary structure, complex turnover regulation and multiple roles of hyaluronan. *Journal of Biochemistry.* **144** (2), 131-137 (2008).

8 Agostinis, C. et al. An alternative role of C1q in cell migration and tissue remodeling: contribution to trophoblast invasion and placental development. *Journal of Immunology.* **185** (7),

4420-4429 (2010).
9 Agostinis, C. et al. Complement Protein C1q Binds to Hyaluronic Acid in the Malignant Pleural Mesothelioma Microenvironment and Promotes Tumor Growth. *Frontiers in Immunology.* **8,** 1559 (2017).
10 Bossi, F. et al. C1q as a unique player in angiogenesis with therapeutic implication in wound healing. *Proceedings of the National Academy of Sciences of the United States of America.* **111** (11), 4209-4214 (2014).
11 Hosszu, K. K. et al. Cell surface expression and function of the macromolecular c1 complex on the surface of human monocytes. *Frontiers in Immunology.* **3,** 38 (2012).
12 Laurent, T. C., Fraser, J. R. Hyaluronan. *Journal of the Federation of American Societies for Experimental Biology.* **6** (7), 2397-2404 (1992).
13 Gaboriaud, C. et al. The crystal structure of the globular head of complement protein C1q provides a basis for its versatile recognition properties. *Journal of Biological Chemistry.* **278** (47), 46974-46982 (2003).
14 Lam, J., Truong, N. F., Segura, T. Design of cell-matrix interactions in hyaluronic acid hydrogel scaffolds. *Acta Biomaterialia.* **10** (4), 1571-1580 (2014).

Figure 1



Tissue minced with a cutter

Cells in a digestion solution
(0.5% trypsin + 50µg/ml DNaseI)
O.N. at 4°C

Incubation for 30 min on 37°C

Centrifugation

250xg

Substitution of enzymatic solution with the Collagenase I for 30 min on 37°C

Filtration of cell suspention through a 100 µm pore filter

Primary tumor cells were seeded in a 12.5 cm^2 flask and cultured in Human Endothelial serum free medium + 10% FBS

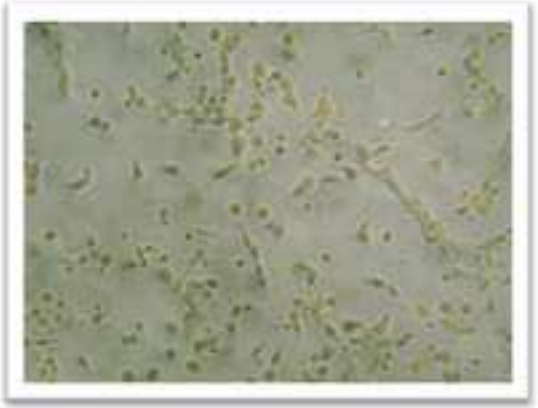
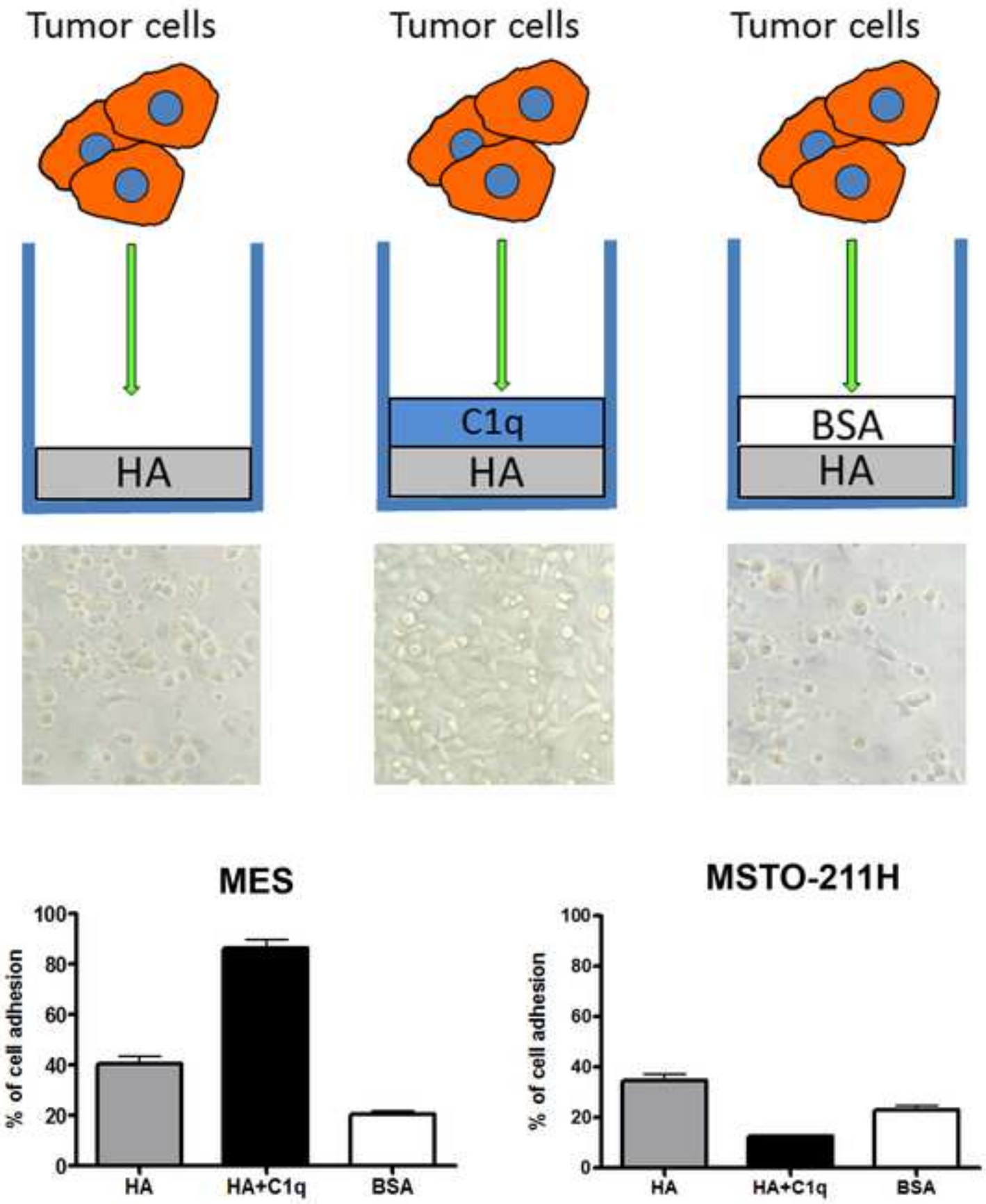


Figure 3



Name of Material/ Equipment	Company	Catalog Number
100 µm pore filter	BD Falcon	352360
Amphotericin B solution (fungizone)	Sigma-Aldrich	1397-89-3
basic FGF	Immunological Sciences	GRF-15595
Calcium Chloride	Sigma-Aldrich	C-4901
Collagenase type I	Worthington Biochemical Corporation, DBA	MX1D12644
D-Glucose	Sigma-Aldrich	50-99-7
DNase I	Roche	10 104 159 001
EDTA	Sigma-Aldrich	60-00-4
EGF	Immunological Sciences	GRF-10544
FAST DiI	Molecular probes, Invitrogen, Thermo Fisher Scientific	D7756
Fetal bovine serum	Gibco, Thermo Fisher Scientific	10270-106
Fibronectin	Roche	11051407001
Flask for cell culture	Corning	430639
Gentamicin solution	Sigma-Aldrich	G1397-10ML
Hank's Balanced Salt Solution (HBBS)	Sigma-Aldrich	H6648
High molecular weight hyaluronic acid	Kind gift by Prof. Ivan Donati	
Human endothelial serum free medium	Gibco, Thermo Fisher Scientific	11111-044
Magnesium Chloride	Carlo Erba	13446-18-9
Medium 199 with Hank's salt	Sigma-Aldrich	M7653
Penicillin-Streptomycin	Sigma-Aldrich	P0781
Time-lapse microscopy	Nikon	Imaging System BioStation IM-Q
Titertek Multiskan ELISA Reader	Flow Labs	
Trypsin	Sigma-Aldrich	T4674

Comments/Description

Sterile, vented

Supplemented with EDTA, Glucose, penicillin-streptamicin, gentamicin and fungizone

Supplemented with EGF (5 ng/mL), basic FGF (10 ng/mL), and 1% penicillin–streptomycin (Sigma-Aldrich)

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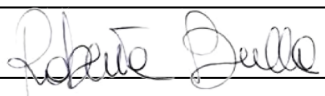
Signature:	
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Dear Alisha DSouza,

we would like to thank the Editor and the reviewers for their comments and suggestions that we have addressed as follow.

Editorial and production comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript has been revised and controlled

2. Please revise lines 77-81, 154-156, 170-172 to avoid previously published text.

The lines have been revised and modified to the best

3. Please revise the title to be more concise.

Done

4. Please provide an email address for each author.

Now the manuscript presents the email address for each author

5. Keywords: Please provide at least 6 keywords or phrases.

There are 6 keywords

6. Please abbreviate liters to L to avoid confusion.

Done

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Done

8. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.

Done

9. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences

wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Done

10. Line 78: Please specify the size of the minced tissue.

Done

11. Line 111: Please describe how to label tumor cells with the fluorescent dye.

A part of text indicating the labbeling procedure has been included.

12. Line 117: For how long are the adherent cells are lysed with Tris-HCl?

Now there is described in the manuscript the lysis method.

13. Figure 1: Please label the x-axis. In the figure legend, please also explain what the right top and bottom parts of the figure show.

Done

14. Figure 2: Please change the unit “ml” to “mL” and include a space between all numerical values and their corresponding units (50 μg/mL, 4 °C, 37 °C, 250 x g). Please replace commercial language (trypsin) with a generic term.

Done

15. Figure 3: Please explain what the bottom panels are.

Done

16. Figure legends: Please move the details of the methodology to the Protocol.

Done

17. References: Please do not abbreviate journal titles.

In reference section the journal titles now are not abbreviated

18. Table of Materials: Please sort the items in alphabetical order according to the name of material/equipment.

Done

Changes to be made by the Author(s) regarding the video:

1. 01:20, 02:16: Please use the micro symbol µ instead of u. Please abbreviate liters to L to avoid confusion. **DONE**

2. 02:16: Please include a space between numerical values and their corresponding units (25 µg/mL, 0.7 mM). **DONE**

3. 05:52: Please include a space between "106" and "cells/mL" (i.e., 1 x 106 cells/mL). **DONE**

4. Please increase the homogeneity between the written protocol and the narration in the video. It would be best if the narration is a word for word from the written protocol text. **DONE**

5. 01:45: The video shows/says "vacuum aspirate the treated wells", which is not mentioned in line 104 of the written protocol. Please revise either the written protocol or the video to be consistent. **DONE**

6. 02:45-02:51: Please remove these to the introduction or results section. **DONE**

7. 02:58-04:05: Such details in the video are not mentioned in the written protocol. **DONE**

8. 04:18-05:30: This part of the video is difficult to follow with the written protocol. Many details in the video are not mentioned in the written protocol. **DONE**

9. Please use the same figures for representative results in the video and the manuscript. **DONE**

10. There is a thin black border on all sides of the frame. This border should be eliminated. **DONE**

11. 0:10-1:04, 6:06-6:47 - The audio/video synchronization appears to be slightly off during these interview segments. This should be corrected. **DONE**

12. 6:47 - To give the end of the video a more conclusive feeling, we recommend putting a title card at the end of the video. Either a repeat of the beginning title card or some production credits would indicate to the viewer that they have indeed reached the end. **DONE**

13. Please upload a revised high-resolution video here:

https://www.dropbox.com/request/DlldWLfX2Uenmly5NmZ6

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors described a method regarding hyaluronan coating and C1q binding as a designed matrix for cancer cell attachment. The approach is conviencing. My concern is that not all cancer cells have C1q and/or HA receptors (CD44 and Hyal-2). Indeed, the data in the Figure 3 shows the status. Please use cancer cell lines, which are devoid of both receptors, for seeding experiments, as negative controls. Also, antibody against C1q, CD44 and Hyal-2 should be used to block the cell attachment. An important concern is that there are no videos showing the behavior of cells upon adherence to the designed matrices. Majority of cancer cells secrete degraded HA and their conformation may be altered. This will alter the scenario of cancer cell adherence to HA/C1q matrix.

We are gratefull to the referee criticism about such a complex issue whose investigation certainly will require a fully dedicated study which is far beyond the scope of Jove. In fact we were asked to simply describe the method of matrices preparation and how to judge cancer cells adhesion on them. At the moment the laboratory is actively involved in the identification and characterizaton of the receptors engaged in HA and/or C1q binding in several cancer cells isolated from human bioptic speciments. The data collected by the ongoing studies will be certainly published on a more appropriate, not methodologic, Scientific Journal.

Major Concerns:

1) The source of hyaluronan, including moleuclar size and DNA and protein-free, should be specified. What's the percentage of HA coated onto microtiter plates?

The source of HA is now specified in the revised manuscript. Regarding the second issue raised by the referee, we determined that the maximal concentration of HA required to saturate the well of the microtiter plate is 50 μg/mL. Therefore measuring the absolute amount of HA actually immobilized in each well will not impact on the scientific relevance of the adhesion data obtained.

2) Does C1q beocme aggregtated upon binding with HA?

Studies regarding the molecular interaction between HA and C1q are in progress, but at the moment there are not definitive data, that anyway are beyond the scope of this methodology paper.

3) Please make videos from time-lapse microscopy for each cell adherence to the matrics and address the differences.

Time lapse microscopy was performed and representative videos have been added to the 'Result' section of the final movie.

Minor Concerns:

1) Please make sure the volume of each person's speech is some what equal in the video.

The volume of each person's speech have been checked and now equalized.

Reviewer #2:

Manuscript Summary:

The protocol is well written and certainly worthy of publication. I recommend that the introduction should be extended to give further context.

The introduction has been extended.

Major Concerns:

no major concerns

Minor Concerns:

-The following references would be useful:

-HA is finding use in immunomodulation please mention in the introduction

-Zamboni, et al. "The Potential of Hyaluronic acid in Immunoprotection and Immunomodulation: Chemistry, Processing and Function" Progress in Materials Science, volume 97, 97-122

-Preparation of HA solutions is of importance see:

-Collins et al (2013)" Hyaluronic Acid Solutions - A processing method for Chemical Modification" Journal of Applied Polymer Science, vol 130, issue 1, pgs 145-152

The references suggested by the referee have been included in the revised manuscript.