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Biomarkers & Applications

Research Article

Corona-Ortega MT, et al. Biomark Applic: BMAP-120.
DOI: 10.29011/BMAP-120. 100020

Intracellular CD16 as a New Phenotype Marker: CD16 $_{Inter}$ in Adherent Leukocytes and Cervical Cancer Cells Can Be Induced by Liposomes Containing Phosphatidylinositol to Be Expressed in Their Cellular Membranes

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Citation: Corona-Ortega MT, Valle-Mendiola A, Soto-Cruz I, Mendoza-Rincón JF, Rangel-Corona R, et al. (2018) Intracellular CD16 as a New Phenotype Marker: CD16 $_{Inter}$ in Adherent Leukocytes and Cervical Cancer Cells Can Be Induced by Liposomes Containing Phosphatidylinositol to Be Expressed in Their Cellular Membranes. Biomark Applic: BMAP-120. DOI: 10.29011/BMAP-120. 100020

Received Date: 01 February, 2018; **Accepted Date:** 12 February, 2018; **Published Date:** 19 February, 2018

Abstract

To evaluate if CD16$^-$ adherent blood leukocytes contain CD16 intracellulary (CD16$_{inter}$) we permeated them and measured its presence by flow cytometry. To determine if they can be induced to express CD16 on their cell membranes they were cultured with the receptor inducer LIPO-PI. We obtained that the great majority of the CD16$^-$ cells had CD16$_{inter}$ and that they became CD16$^+$ after a short incubation with LIPO-PI. Our results thus hint that there might not exist different subgroups of CD16$^-$ and CD16$^+$ monocytes but leukocytes in different stages of activation.

To evaluate if cervical cancer cells contain CD16$_{inter}$ the cervical cell line INBL was permeated and CD16 presence determined by flow cytometry. We obtained that INBL cells had CD16$_{inter}$ and in a similar way as with leukocytes they expressed CD16 in their membranes when cultured for short times with LIPO-PI. Taking into consideration that endocervical columnar epithelial cells express CD16 and not the exocervical ones our results hint to the endocervical origin of INBL. We propose CD16$_{inter}$ as a new phenotype marker.

Keywords: CD16; Cervical Cancer; Epithelial Cells; Leucocytes; Liposomes; LIPO-PI; Phenotype Marker; Phosphatidylinositol

Inroduction

Fc Receptors (FCR) have been identified in leukocytes where they play an important role in the immune response [1-4]. CD16 also known as FcRIII is expressed by almost all types of leukocytes and performs a wide range of activities [5-8]. Several authors have postulated the existence of different sub-populations of monocytes depending on their capacity to express membrane CD16 receptors [9-11]. Other authors have published that CD16$^-$ blood monocytes become CD16$^+$ in culture [12,13]. In this last respect either the expression of CD16 is a product of differentiation of one subgroup into another or the membrane expression of an already existing internal receptor.

We proceeded to obtain CD16$^-$ blood mononuclear adherent leukocytes to determine if there existed an internal expression of this receptor and then used a liposome (LIPO-PI) capable to induce membrane expression of internal receptors [14] to evaluate if this receptor could be induced. CD16 has also been identified in epithelial cells [15,16] and proposed to have important

immunological functions. A role as an antigen transportation from the external media into the underlying lymphoid tissue was proposed (Kraehenbuhl 1997, Neutra 2001) [17,18] as well as that of viruses and even being the cause for viral infection when they malfunction [15].

The prevailing theory of the origin of Cervical Cancer (Ceca) cells asserts that these tumors arise from transformed basal cells in stratified exocervical epithelium [19,20]. Ceca has also been classified as a metaplastic disease because most tumors are generated in the transformation zone from endocervix columnar epithelium [21,22]. It is then unclear whether the origin of those cells is in the endo or the exocervix. CD16 has been found in columnar epithelium of the stomach [15] and strongly expressed in the endocervix and metaplastic tissue but not on the exocervix [23]. In order to determine if Ceca cells could have arisen from the endocervix we evaluated intracellular CD16 presence in a CeCa cell line INBL and its possible induction for membrane expression by LIPO-PI.

Materials and Methods

Reagents

Hystopaque® (1.077 g/ml) density gradient, crystal violet, egg yolk phosphatidylcholine, L-α-phosphatidyl-D-myo-inositol 4,5-diphosphate dioctanol (PI (4,5) P2), cholesteryl chloroformate and spermidine base were acquired from Sigma (Sigma Chem, St Louis, MO, USA). RPMI medium was obtained from Microlab (Microlab, Mexico City, Mex) and Fetal Bovine Serum (FBS) from Hyclone (Hyclone, Logan UT, USA). A fluorescent antibody against CD16, (FcγRIII)-Per CP, was purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., Dallas, TX, USA).

Preparation of LIPO-PI

Liposomes with PI (4,5) P2 (LIPO-PI) were formed as previously described (Corona 2016) Briefly: The cationic lipid Spermidine-Cholesterol (SpeCho) synthesized by direct reaction of cholesteryl chloroformate and spermidine base was mixed at a 1:1 molar ratio with egg yolk Phosphatidylcholine (PC) with or without 500 fG/mL of PI (4,5) P2. The mixtures of lipids (10 µmol) were dissolved in chloroform and dried under nitrogen at reduced pressure. Liposomes were produced by hydration of the resulting thin lipid film in phosphate buffer saline (PBS) using three 5-second sonication cycles followed by a 30 second resting period using an Avanti Sonicator (Avanti Polar Lipids, Inc., Alabaster, AB, USA). The liposomes without PI (4,5) P2 (LIPO) or with this Inositol (LIPO-PI) were finally re-suspended in PBS.

Cell Culture

The human cell line from Ceca INBL was established in our laboratory from a patient with cervical carcinoma [24]. The cells were routinely sub-cultured at 37°C with 5% CO_2 in culture media containing RPMI supplemented with 10% FBS.

Peripheral blood was collected with an anticoagulant by venipuncture of the medial cubital vein from normal volunteers. Leukocytes were carefully deposited on Hystopaque and cell separation was obtained by centrifugation at room temperature for 30 minutes at 400 xg. The mononuclear cells at the interface were carefully collected and washed twice with RPMI by centrifugation at room temperature. The cells were cultured for 24 h and the Adherent Cells (ABL) were separated from non-adherent by carefully washing 3 times with RPMI. ABL cells were recovered and washed two more times before use.

Membrane and Intracellular CD16 Receptors

To detect the presence of membrane CD16 the cells were fixed with paraformaldehyde at 1.1%. After washing the cells with RPMI by centrifugation a fluorescent anti-CD16 was added at a concentration of 5 µg/mL and incubated with culture media for 30 min in the dark at room temperature.

To evaluate intracellular content of CD16 ($CD16_{inter}$) the cells were permeated with cold methanol for 15 min at -20°C and washed twice with cold PBS prior to incubation for 30 min with anti-CD16. All the cells were then washed twice with PBS and a flow cytometer was used to detect fluorescence.

Results

$CD16^-$ Blood Adherent Cells Were Induced to Express CD16 by LIPO-PI

We obtained as expected that by cytometry all the ABL cells were $CD16^-$ (Figure 1a) and that when they were permeated a significant number of cells were positive for $CD16_{inter}$ (Figure 1b). One hundred thousand ABL cells were also cultured for 30 min in the presence of LIPO, LIPO-PI and 500 fG/mL of PI (PI(4,5) P2).

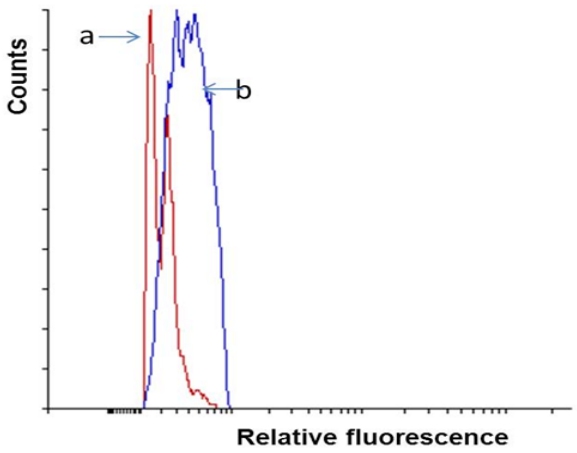

Figure 1. Intracellular CD16 in ABL cells. ABL cells were permeated and evaluated for intracellular CD16 by a fluorescent anti-CD16 by flow cytometry. a) No permeated cells and b) Permeated cells.

Cells without antibodies and without treatment were used as negative controls. We obtained by cytometry that only the cells cultured with LIPO-PI significantly expressed CD16 (Figure 2).

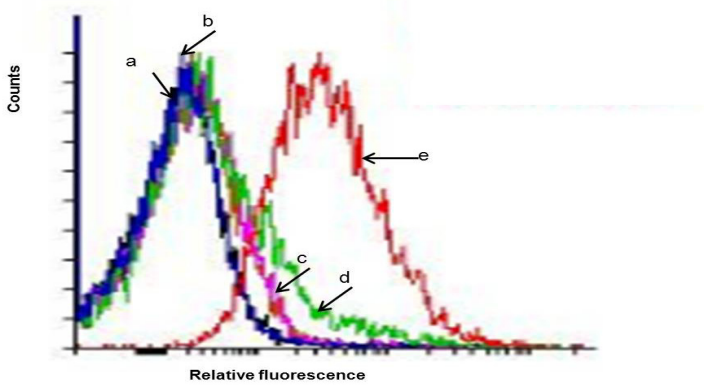

Figure 2. Induction of CD16 on ABL cells by LIPO-PI. ABL cells were cultured for 30 min and evaluated for intracellular CD16 by a fluorescent anti-CD16 by flow cytometry. a) Autofluorescence; b) No treatment; c) 500 fG/mL of PI(4,5)P2; d) LIPO and e) LIPO-PI.

INBL Cells Contain CD16$_{inter+}$ and were Induced to Express Membrane CD16 by LIPO-PI

By cytometry we obtained that INBL cells were CD16^{-} (Figure 3a) but when they were permeated there was a significant number of cells that were positive for CD16 (Figure 3b). One million INBL cells were also cultured for 30 min in the presence of LIPO, LIPO-PI and 500 fG/mL of PI (4,5) P2. Cells without antibodies and without treatment were used as negative controls. We obtained by flow cytometry that only the cells cultured with LIPO-PI significantly expressed CD16 (Figure 4).

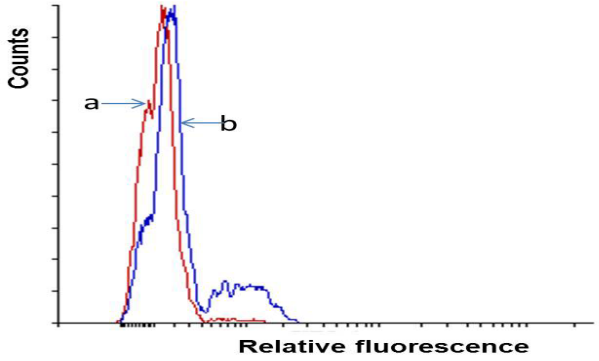

Figure 3. Intracellular CD16 in INBL cells. INBL cells were permeated and evaluated for intracellular CD16 by fluorescent anti-CD16 by flow cytometry. a) No permeated cells and b) Permeated cells.

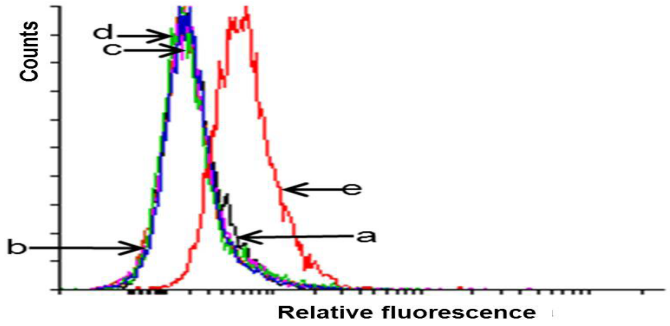

Figure 4. Induction of CD16 on INBL cells by LIPO-PI. INBL cells were cultured for 30 min and evaluated for intracellular CD16 by a fluorescent anti-CD16 by flow cytometry. a) Autofluorescence; b) No treatment; c) 500 fG/mL of PI(4,5)P2; d) LIPO and e) LIPO-PI.

Discussion

In our culture conditions most ABL cells were $CD16^-$ and presented this receptor intracellularly ($CD16_{inter}$). Other monocyte purification techniques would have to be tried to generalize our results but the fact that almost all adherent ABL cells presented $CD16_{inter}$ point into that direction. It would be interesting to evaluate the possible existence of monocytes in disease that may not express CD16 neither internally or externally or with a damaged cellular trafficking mechanism that precludes its membrane expression.

We propose the use of CD16 intracellular ($CD16_{inter}$) or CD16 membrane ($CD16_{ext}$) as new tool for leucocyte phenotype. Even though we obtained that INBL a CeCa cell line contains $CD16_{inter}$ we cannot certify that all CeCa cells in their different stages of differentiation do for that purpose other cell lines and in situ determinations would have to be performed. There are other epithelial metaplastic transition areas in the body that are known to be prone for cancer [25,26] we think it would be interesting to evaluate if their columnar epithelial cells also have $CD16_{inter}$.

Columnar epithelial cells are known to transport external viral particles to activate the immunological defense and that when they malfunction to be the cause for viral infection [15]. In consequence we could speculate that the HPV virus strongly associated with CeCa could use the same pathway for infection. It would be interesting to identify if the CD16inter expressing cells in the endocervix

have the capacity to transport HPV particles and for its possible malfunction when forced to transform into stratified epithelium. This possibility would be a better explanation for HPV infection in CeCa rather than to assume that basal cells, that are not present in the transformation zone, are responsible. We think that a better identification of the cancer-producing cell associated with CeCa and its mechanisms of viral infection could redirect research in this area as well as clinical protocols.

M cells have been identified in columnar epithelium and propose to participate in the immunological response [27]. They have been found as antigen presenting cells and to transport viral particles [28,29]. It would be interesting to evaluate if M cells could express Fc receptors and in particular CD16. Even though we could induce CD16 membrane expression on ABL and CeCa cells with LIPO-PI it would be interesting to determine whether intracellular CD16 is active as other receptors have been found to be [30] and the role it could play in the immunological response. In this discussion we advanced two new hypotheses. First that $CD16^-$ monocytes have $CD16_{inter}$ and thus the possible inexistence of $CD16^-$ cells but only cells in different stages of activation and second that Ceca might have an endocervical origin because both contain $CD16_{inter}$. We propose the use of $CD16_{inter}$ as a new marker for cellular phenotype.

Conclusion

We propose the use of CD16 intracellular ($CD16_{inter}$) and CD16 membrane ($CD16_{ext}$) as a new tool for leucocyte and epithelial cell phenotype. We provide evidence that $CD16^-$ leukocytes and in particular monocytes are $CD16_{inter}$ positive. Evidence is also given that cervical cancer cells could have a columnar epithelial origin.

Acknowledgements

The authors acknowledge support from a DGAPA, UNAM IN219315 grant. We thank M. Sc. Margarita Cruz Millán for assistance with image and data processing and José Chavarría for excellent technical assistance.

Declaration of interest

The authors report no conflicts of interest.

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