VEGF-R1 activation increases survival of purified retinal ganglion cells

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[1]Institut de la Vision – UMR S 968, INSERM, Université Pierre et Marie Curie, CNRS-UMR_7210, CHNO des Quinze-Vingts, Paris, France.

[2]Institut Fédératif de recherche 65, Hôpital *Saint-Antoine*, PARIS, France.

3CHNO des Quinze-Vingts, Fondation Adolphe de Rodchild

INTRODUCTION

Retinal ganglion cell (RGC) damages are the ultimate and common process characterizing degenerative retinopathies, like glaucoma. In these diseases, no treatment is yet available to directly target RGC degeneration. Previously, we reported that a retinal conditioned medium was able to increase adult RGC survival in pure cultures[1]. Our aim was to therefore identify RGC neuroprotective molecules contained in this medium.

Here, we found that endogenous VEGF could be a good candidate for RGC neuroprotection strategies

METHODS

Retinal Mixed cultures from adults rats

Mixed cultures from adult rat retinas were performed, as previously described by Fuchs et al. (2005)[1]. Cells were cultured in DMEM+10% FCS medium, changed one time until confluence obtained after 10-12 DIV. Conditioned medium from mixed cultures(CM-M) were then prepared by a 48h incubation of either the serum-deprived medium (Neurobasal-glutamine) or the high nutritive medium (Neurobasal-glutamine+B27).

Retinal glial and microglial cultures from adults rats

Glial cultures were obtained with the similar cell suspension as used for mixed cultures. After 48h, the culture medium was changed and cells were vigorously rinsed to detach neurones, which are less adherent to coverslips than glial cells. At 10-12 DIV, confluent cultures are exclusively composed by a glial monolayer. Conditioned medium from glial cultures (CM-G) was prepared by a 48h incubation of the neurobasal-glutamine medium. Microglia cell suspensions were obtained by a gently shacking of confluent mixed cultures (12-14 DIV). Microglia were then directly seeded in a neurobasal-glutamine for 48h incubation to obtain a microglial conditioned medium (CM-MG).

RGCs pure cultures from adult rats:

RGCs were purified by immunopanning protocol previously described[2]. They were cultured for 6 days *in vitro* (DIV) in a serum deprived medium or in high nutritive medium . A CalceinAM labeling for viable RGC was performed at 6 DIV to evaluate the RGC survival. A counting of RGC was then realized on the full wells from 96-well plates.

Retinal explants from adult rats:

Retinas were prepared as described by Vallazza-Deschamps et al. (2005)[3]. They were cultured in high nutritive medium for 4 DIV. RGCs death were induced by incubation of 100µM NMDA all along of the culture. Brn-3a-positive RGCs were counted on full flat-mounted retinas, using automatic counting (using Metamorph software).

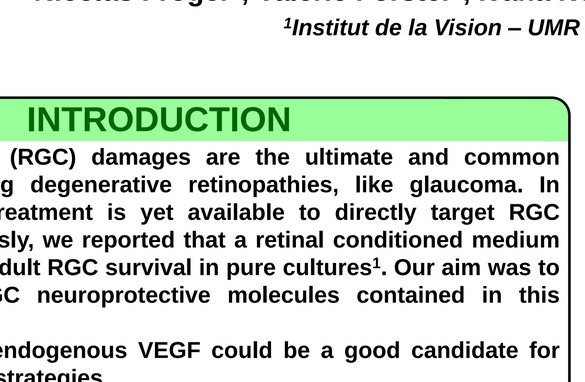
Growth factors measurments

Rat Growth factors amounts were measured in culture supernatants by Luminex technology and rat VEGF (164) amounts were then measured by ELISA kit (R&D system) according instructions.

RESULTS

I- Effect of conditioned medium (CM) harvested from Mixed retinal cell cultures on RGC survival

Characterization of mixed retinal cell cultures

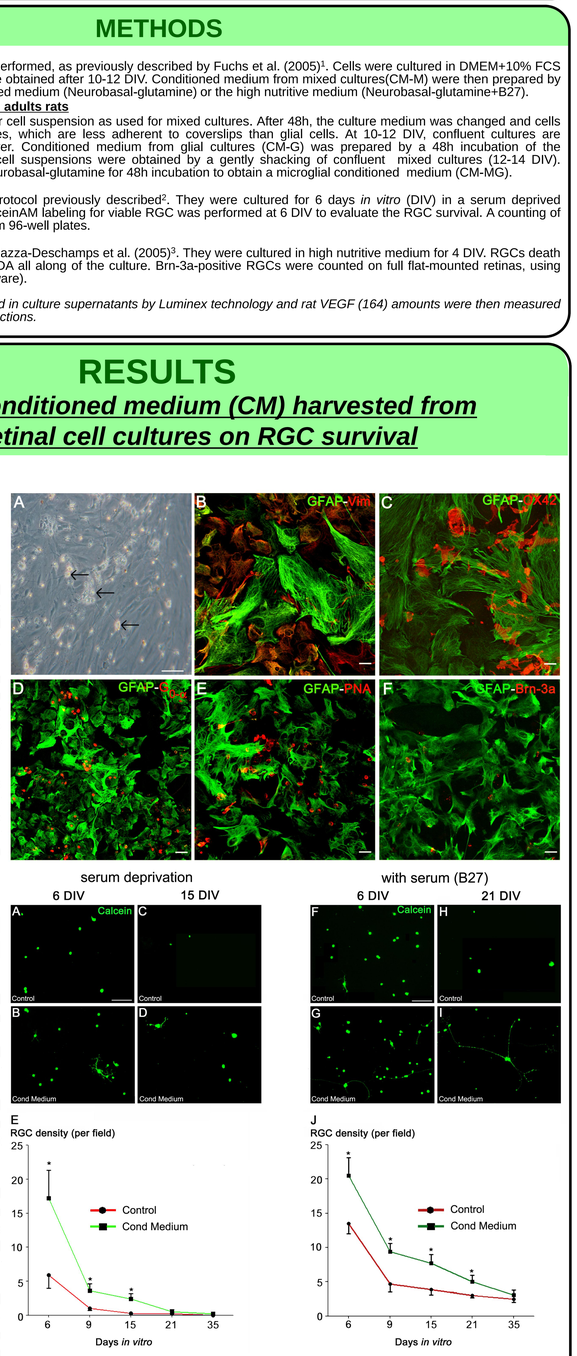


A: Confluent mixed retinal cell cultures at 10-12 DIV. Cultures were constituted by differentiated neuronal cells growing upon a glial monolayer. Scale bar: 50µm

B-C: Immunostaining of glial cells in retinal mixed cultures with Vimentin (for Müller cells, in red; B) or OX42 (for microglia, in red; C) versus GFAP (in green). Note the high density of microglia. Scale bar: 20 µm

D-F: Immunostaining of neuronal cells in retinal mixed cultures with G0-a (for bipolar cells, red; D) PNA (for cone photoreceptors, red; E), or Brn-3a (for RGCs, red; F), versus GFAP (in green). Scale bar: 20µm

Long-term neuroprotective effect of conditioned medium from mixed cultures on RGCs



A-D: Calcein-positive RGCs cultured under the serum-deprivation, in control medium (Neurobasal-glutamine; A, C) or in conditioned medium (B, D), after 6 DIV (A, B) or 15 DIV (C, D).

E: Densities of alive RGC, cultured in control medium (red line) or in conditioned medium (green line) at different days *in vitro.*

F-I: Calcein-positive RGCs cultured with serum, in control medium (Neurobasal-glutamine medium plus 2% B27; F, H) or in conditioned medium (G, I) after 6 DIV (F, G) or 21 DIV (H, I).

J: Densities of alive RGCs cultured in control medium (neurobasal-glutamin+B27 supplement; red line) or conditioned medium (green line) after different days *in vitro.*

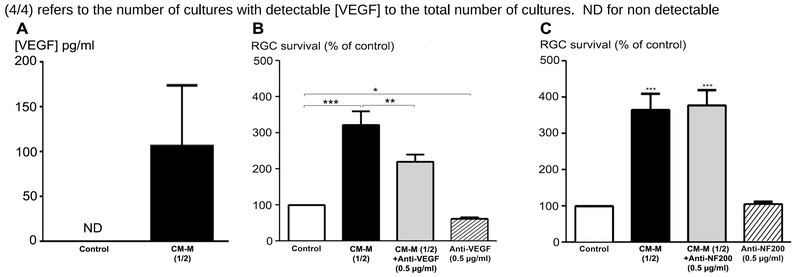
Conditioned medium harvested from mixed retinal cell cultures displays a robust and long-term neuroprotective effect on pure cultured RGC

II- VEGF production in retinal mixed cultures

Luminex technology	Growth factors (pg/ml)			pg/ml; n=4
	VEGF-A	BDNF	GM-CSF	G-CSF
Control Medium (neurobasal-glutamine)	ND	ND	ND	ND
Conditioned medium (retinal mixed cultures)	86.5±13.2 (4/4)	ND	ND	ND

VEGF-A (164) is present in retinal mixed cultures

(4/4) refers to the number of cultures with detectable [VEGF] to the total number of cultures. ND for non detectable



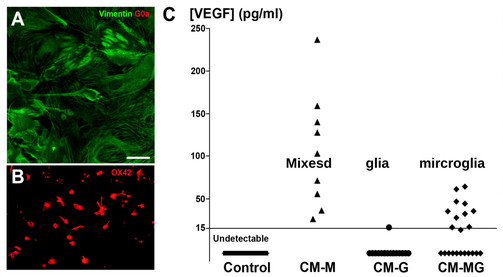
VEGF-A (164) produced by retinal mixed cells, contributes to the protective effect of conditioned medium on RGCs

A: VEGF-A measurement by ELISA in conditioned medium from retinal mixed culture (CM-glia). ND for no detectable.

B: Incubation of anti-VEFG-A antibody significantly reduced the neuroprotective effect of CM-glia on RGCs.

C: Incubation of anti-NF200 polyclonal antibody did not affect the neuroprotective effect of CM-glia on RGCs

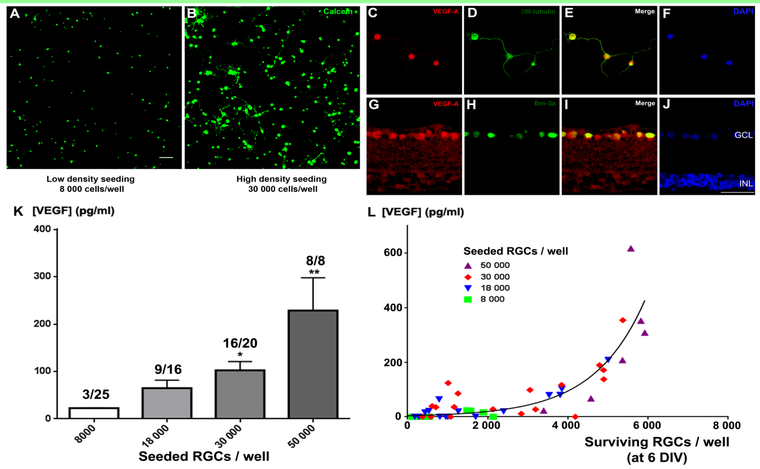
III- VEGF production by retinal microglial cells



VEGF-A (164) is measurable in pure cultures of adult retinal microglial cells but not of glial cells A: Immunostaining of pure retinal glial cultures from adult rats. Glial cells are labeled with Vimentin (green), whereas bipolar cells, labelled with G0a, are absent. Scale bar: 50 µm. B: Immunostaining with OX-42 of microglial pure cultures from adult rats

C: VEGF-A (164) amounts measured by ELISA, in control medium (Neurobasal-glutamine), conditioned medium from mixed retinal cell cultures (mixed), from retinal macroglial cell cultures (glia) or finally from retinal microglial cell cultures(microglia). I

IV- VEGF-A is produced by pure retinal ganglion cells in cultures



RGC survival is correlated to VEGF-A (164) production

A-B: Alive RGCs stained with calcein at low density seeding (A) and high density seeding (B). RGC morphology appears larger in size with an higher neurite development at high density than at low density seeding. Scale bar: 50 µm

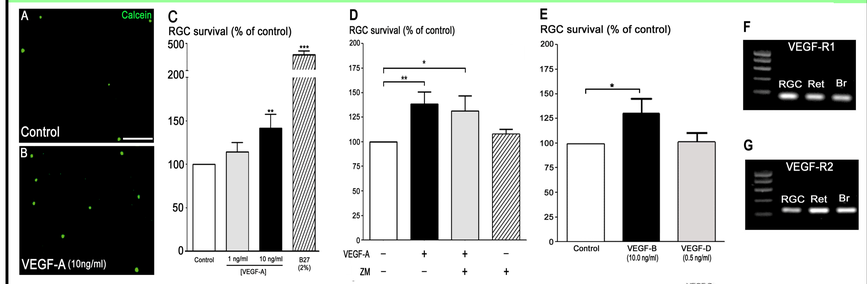
C-J: VEGF expression in cultured RGCs (C-F) and in retinal sections (G-J). Scale bar: 50 µm

K: VEGF amounts measured, according the initial RGC seedings. n/N refer to the number of experiments with measurable [VEGF] (n), compared to the total experiments (N).

L: Graphe representing the VEGF amounts measured in RGCs cultures in function surviving RGCs measured at 6 DIV.

VEGF is an autocrine and paracrine factor for RGC survival

V- Mechanisms of VEGF-elicited RGC survival



Recombinant VEGF-A (164) stimulates the RGC survival in cultures. This effect is mediated through the VEGF-receptor1 stimulation.

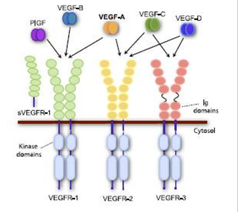
A-B: Calcein-positive RGC cultured for 6 DIV either in control medium or in presence of VEGF-A (164).

C: Effect of recombinant VEGF-A (164) on RGCs survival, evaluated by counting of alive RGCs. B27 supplement is used for a positive control (high-nutritive conditions).

D: Addition of ZM 323881 (5nM), a selective VEGF-R2 antagonist (ZM) with the recombinant VEGF-A (10ng/ml)

E: Effect of recombinant VEGF-A (164) (1ng/ml and 10ng/ml) on RGCs survival, evaluated by counting of alive RGCs 10ng/ml F-G)

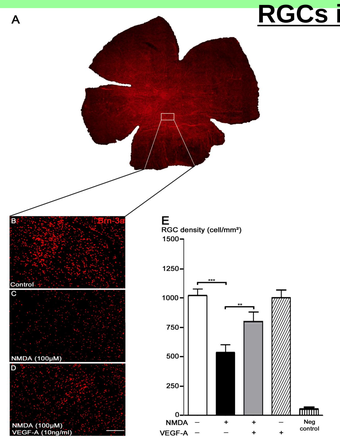
F-G: Gene amplification (PCR) of VEGF-R1 (F) and (VEGF-R2 (G) performed on total cDNAs from freshly purified rat retinal ganglion cells (RGC) and rat full retina (Ret), whereas rat brain tissue (Br), vas taken as positive control. These data revealed a high expression of these two receptor transcripts in pure RGCs



From Ruiz de Almodovar, 2008[4].

Pharmacology of VEGFs

V- VEGF prevents NMDA-elicited glutamate excitotoxicity of RGCs in retinal explants



NMDA induced a RGC loss in retinal explants, which is partially suppressed by VEGF-A (164)

A: Digitalized reconstruction of a whole flat-mounted retinal explant after 4 days in culture, immunolabeled with Brn3a specific marker

B-D: Enlarged fields from flat-mounted retinal explants acquired with the automated platform, showing Brn-3a-immunopositive RGCs in a control untreated condition (Control; A) after 100µM NMDA application (NMDA, B), after co-application of 100µM NMDA with 10ng/ml VEGF-A (C) for 4 days *in vitro*. The scale bar represents 100µm.

E) Quantification of RGC densities by automatic counting on whole flat-mounted retinal explants either in control untreated condition (Control, white bar) and after application of either 100µM NMDA (black bar), or 100µM NMDA plus 10ng/ml VEGF-A (grey bar) or finally VEGF-A alone (Oblique hatched bar). The negative control (vertical hatched bar) represents the quantification of unspecific labeling provided by the incubation of the secondary antibody (goat anti-mouse IgG AlexaFluor® 594) alone.

CONCLUSION

- **Mixed retinal cells in culture produce VEGF.**
- **This VEGF is likely to come from microglial cells, which produce VEGF in pure cultures.**
- **VEGF contributes to the RGC survival effect induced by retinal conditioned media.**
- **Pure RGCs produce VEGF and this production is correlated to the cell survival.**
- **Recombinant VEGF directly stimulates the RGC survival through VEGF-R1 stimulation.**
- **VEGF can prevent the NMDA-elicited excito-toxicity of RGCs**
- **VEGF is therefore an autocrine/paracrine factor for RGC survival**

References

[1]Fuchs C et al. (2005). Retinal-cell-conditioned medium prevents TNF-alpha-induced apoptosis of purified ganglion cells. *IOVS* **46:2983-2991.**

[2]Barres B et al. (1988) Immunological, morphological, and electrophysiological variation among retinal ganglion cells purified by panning. *Neuron* **1: 791-803.**

[3]Vallazza-Deschamps et al. (2005) Excessive activation of cyclic nucleotide-gated channels contributes to neuronal degeneration of photoreceptors. *Eur J Neurosci* **22:1013-1022**

[4]Ruiz de Almodovar C et al. (2009) Role and therapeutic potential of VEGF in the nervous system. Physiol Rev. 89(2):607-648.

Supports

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Address for correspondence: nicolas.froger@inserm.fr