Supplement

Materials and Methods

Animal models of cerebral hypoperfusion and therapeutic induction of collateral growth

For evalution of the effects of G-CSF on cerebral arteriogenesis, two different models of cerebral hypoperfusion were used, three vessel occlusion (3-VO) in rats [1,2] and left common carotid artery occlusion (CCAO) in mice (Fig. 1A).

3-VO includes bilateral vertebral artery occlusion followed by ligation of the left common carotid artery as described previously [1,3]. To show relevance of 3-VO in rats and CCAO in mice for the induction of permanent hypoperfusion, CCAO was performed in an additional rat group (n = 6) and compared with 3-VO. Cerebral blood flow was measured by laser Doppler flowmetry.

Anaesthesia was initiated with 50 mg/kg ketamine/4 mg/kg xylazine i.p. in rats and 100 mg/kg ketamine/10 mg/kg xylazine i.p. in mice and maintained with 1-2% isoflurane in oxygen p.i.. In all groups Ropivacaine (5 mg/kg; Naropin®, AstraZeneca) was infiltrated into the wounds against pain.

Cerebrovascular Reserve Capacity (CVRC)

In isoflurane anaesthesia, cerebrovascular reactivity measurement was initiated by injection of 30 mg/kg acetazolamide (ACZ, Diamox ®, Sanofi Aventis) intravenously in rats as described previously [2] and into the peritoneal cavity in mice. Changes of CBF were continuously monitored by LDF measurement.

For arterial blood gas analysis, blood samples were taken in rats from the cannulated femoral artery before and after CVRC measurement.

Postmortem Latex Angiography

To visualize the angioarchitecture of the circle of Willis, postmortem latex angiography was carried out in maximal vasodilation as described in rats [1,3]. In mice, a lethal dose of 1 mg/kg adenosine was injected into the cannulated abdominal aorta. To facilitate venous outflow, the right cardiac auricle was incised after thoracotomy. After maximal vasodilation, 5.5-6 mL/kg of warm (37°C) latex milk (Chicago Latex Products, no. 563) were injected at 150 mmHg. Animals were placed on ice for 15 minutes to harden the latex. Vessel diameters of all arteries of the circle of Willis were measured under the microscope (Stereomikroskop Leica MZ6).

Immunohistology

Brain samples were embedded in paraffin after 4% PFA fixation. 5μm sections were prepared for haematoxylin–eosin staining and immunohistochemistry. Macrophages (1:200, PA43002; Little Chalfont, Buckinghamshire, UK) were identified with an ED-1 (CD68) antibody (1:100, BM 4000; Acris) staining. Nuclei were counterstained with Hoechst 33342 (Molecular Probes Inc., Eugene, OR, USA). A total of 12 sections from each animal were analyzed in a blinded approach by three independent investigators.

Monocyte Migration Assay

After cultivation in RPMI 1640 with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin at 95% relative humidity and 5% CO_2 at 37ºC, human monocytic THP-1 cells (German Resource Centre for Biological Material) were rendered quiescent by serum-starvation overnight [4].

THP-1 cells in transwell cell culture chambers with a gelatin-coated (0.2%) polycarbonate membrane (8 μm pores) then migrated towards either 10 ng/mL or 100 ng/mL MCP-1 as positive control; 25 or 50 ng/mL GM-CSF; 25 or 50 ng/mL G-CSF; 25 or 50 ng/mL M-CSF. Four hours later, cells that had migrated to the lower surface of the filters were determined

microscopically. Four randomly chosen HPFs (320x) were counted per filter. Migration assays were performed three times in triplicates.

Supplemental Results

Table

	post mortem latex angiography – diameter		cerebrovascular reserve capacity
	PCA ipsi [µm]	PCA contra [µm]	CVRC [%]
untreated	159 ± 40 ‡ (n = 6)	167 ± 14 ‡ (n = 6)	18 ± 6 †‡ (n = 8)
1d 3-VO control	-	-	0 ± 10 * (n = 3)
7d 3-VO control	209 ± 7 * (n = 6)	208 ± 11 * (n = 6)	3 ± 4 * (n = 6)
7d 3-VO 40µg/kg dieb. alt. G-CSF	271 ± 57 *‡ (n = 6)	252 ± 28 *‡ (n = 6)	19 ± 12 †‡ (n = 6)
7d 3-VO 50µg/kg/d G-CSF	251 ± 14 (n = 6)	228 ± 23 (n = 6)	14 ± 10 (n = 5)
7d 3-VO 150µg/kg/d G-CSF	217 ± 38 § (n = 6)	205 ± 28 § (n = 6)	1 ± 5 § (n = 5)
7d 3-VO 40µg/kg dieb. alt. GM-CSF	280 ± 20 *‡ (n = 6)	250 ± 16 *‡ (n = 6)	18 ± 12 †‡ (n = 6)

values are means ± SD;
* $p \leq 0.05$ = compared to untreated;
† $p \leq 0.05$ = compared to 1d 3-VO control;
‡ $p \leq 0.05$ = compared to 7d 3-VO control;
§ $p \leq 0.05$ = compared to 7d 3-VO G-CSF at 40µg/kg dieb. alt.

Table. G-CSF and GM-CSF augmented cerebral arteriogenesis after 3-VO and G-CSF dose determination

References

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